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EXPERIMENTAL STUDIES ON MITOMYCIN C
3. COMBINED THERAPY*

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In our previous papers, "Experimental Studies on Mitomycin C, 1" and "Experimental Studies on Mitomycin C. 2. Cytotoxicity" published in this journal (63, 64), we reported that intermittent application of Mitomycin C induced lesser toxic effects than consecutive therapy, and that the inoculation of nucleated cells to animals receiving near-toxic doses of Mitomycin C prevented leucopenia and decreased the mortality rate of animals. It was also disclosed that blockade of the R.E.S. in rats given Mitomycin C by saccharated iron oxide resulted in accentuated leucopenia, while activation of this system by hematoporphyrin prevented it. The clinical trials conducted by Shimada *et al.* (48, 49), Shiraha *et al.* (50, 51), Miller and McLemore (35), Wakaki (75), and others, have confirmed our experimental findings. In the present study, an attempt was made to increase antitumor activity of Mitomycin C by supplementing it with various oncostatic preparations in order to achieve the maximal possible inhibitory effect of this antibiotic. The following preparations were used in the combined therapy with Mitomycin C: 2-Deoxy-D-glucose; 2,3-Enediol-D-glucoheptono-1,4-lactone; 8-Azaguanine and 6-Mercaptopurine. All these four substances belong to the group of antimetabolites, all of them exerting oncostatic activity of various degrees. Since the intrinsic nature of the oncolytic activity of Mitomycin C is still unknown, and so far no enzymic system in which Mitomycin C is involved seems to be discovered, it was presumed that by combining

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an oncolytic and an oncostatic drug, a synergestic action of the two drugs might be achieved.

2-Deoxy-D-Glucose and Mitomycin C

Cramer and Woodward (15) have shown that 2-DG competes with glucose in the yeast fermentation system and thereby inhibits fermentation of glucose. It was also demonstrated by Woodward and Hudson (79) that 2-DG inhibits the anaerobic and aerobic glycolysis by rat tumor slices. Wick *et al.* (77) stated by 2-DG, by inhibiting the transfer and oxidation rates of glucose, acts as a metabolic block for glucose. Brown and Bachrach (6) investigated the effect of 2-DG on rat blood glucose. Sokoloff *et al.* (60, 61) revealed that the glucose analog decreased the total and nuclear DNA content in rat carcinoma G-175. They reported that this analog, added to a meat ration in 2%, 5% and 10%, inhibited the growth of Crocker rat carcinoma (59). Ely (19) reported that the subcutaneous administration of 2-DG increased the survival time of mice bearing Krebs ascites carcinoma. Sokoloff *et al.* (62) administering 2-DG orally to mice and rats found a definite inhibition of rat carcinoma G-175, and mouse sarcoma 180. There was also retardation of young rat growth when 2-DG was given. Laszlo *et al.* (29) showed that oral administration of the analog to advanced L-1210 mouse leukemia resulted in the prolongation of the life span by 20-30 per cent and reduction in tumor size. Ball *et al.* (1) reported that 2-DG in a dose 1.0 mg/kilo weight inhibited Walker 256 carcinoma. Laszlo *et al.* (30) showed that 2-DG induced a regression of the Bashford M63 solid tumor, but no prolongation of survival time was observed. On the other hand, at 12 gm/kilo dose the local tumor at the site of leukemic inoculation was completely resorbed. From this review of the literature on 2-Deoxy-D-glucose, it appears that the analog exerts, if administered in relatively large doses, moderate oncostatic activity associated primarily with the inhibition of glycolysis. Since Mitomycin C has no effect on glycolysis, according to Sokoloff (65) and others, it was decided to apply the combined treatment, with 2-DG and the antibiotic.

Experimental. From the observations of Sugiura and Stock (69, 70), Sugiura (71, 72), Sokoloff *et al.* (63) and others, it was established that the total dose of Mitomycin C, 18.0 mg/kilo wt./mouse and 10.0 mg/kilo wt./rat is necessary to inhibit completely, or almost

completely various transplanted tumors. This dose, given consecutively in seven days results in loss of weight and other toxic manifestations in animals so treated (Sternberg *et al.*, 68). The intermittent therapy reduced to some extent the toxicity induced by such total doses of Mitomycin C, but not sufficiently for proper evaluation of the additive effects of 2-DG. For this reason, smaller total doses of the antibiotic, 14 mg/kilo wt./mouse and 8.0 mg/kilo wt./rat were used in the present combined treatment. This total dose, applied intermittently, produces only a partial, yet considerable inhibition of tumor transplant of 48 hours.

Mouse sarcoma 180, Ehrlich mouse carcinoma, and Crocker rat carcinoma were investigated on the additive inhibitory effects of Mitomycin C and 2-DG. Mitomycin C was injected intraperitoneally, every second day, and 2-DG was given orally, by pipette, daily, 400 mg/kilo wt./mouse and 300 mg/kilo wt./rat, during the whole course of Mitomycin C therapy. Since 2-DG is eliminated rapidly from the animal organism, the daily dose was divided in two. One half was administered in the mornings and the rest in the late afternoons.

Tables I, II and III summarize the results of these tests.

Analysis of results. As was previously pointed out, Mitomycin C does not interfere with glycolysis of tumor tissue. On the other hand, 2-Deoxy-D-glucose is a powerful antagonist of glucose, and thus indirectly influences the growth processes in tumor cells, by inhibiting them. The results of our experiments summarized in Tables I, II and III seem to indicate a definite additive inhibitory effect of 2-DG to the oncolytic activity of Mitomycin C. Using the subtoxic doses of Mitomycin C administered intermittently, it was possible to obtain, when the antibiotic was used alone, only partial inhibition of mouse sarcoma 180, Ehrlich mouse carcinoma and Crocker rat carcinoma. In all three series of experiments the growth processes, although greatly inhibited, still continued. At the end of a fifteen day period of therapy, the average tumor weight was for sarcoma 180—934 mgs., for Ehrlich carcinoma 758 mgs., and for Crocker rat carcinoma 1124 mgs.

The antimetabolite of glucose, 2-DG, in its turn, if administered alone, in large amounts, orally, did exert moderate influence on tumor growth. The inhibition rate for sarcoma 180 was about 25%; for Ehrlich carcinoma about 30%; and for Crocker rat carcinoma about 35%. When the combined therapy was applied, the inhibition

TABLE I

ADDITIVE INHIBITORY EFFECT OF 2-DEOXY-D-GLUCOSE AND MITOMYCIN C ON MOUSE SARCOMA 180

Mitomycin C total dose 14 mg/kilo wt./mouse, given intraperitoneally in fourteen days. 2-DG, total dose 5.6 gm/kilo wt./mouse, orally, in fourteen days.

Group A.—Mitomycin C alone
Group B.—2-DG, alone
Group C.—Mitomycin C and 2-DG
Group D.—Control, no treatment

Each group of 25 mice

Groups	Initial av. wt. gms.	Final av. wt. gms. after 15 days	Tumor av. wt. mgs. after 15 days	Mortality Rate %
Group A Mitomycin C total dose 14 mg/kilo wt. intraper.	21.3	23.5	934	0
Group B 2-DG total dose 5.6 gm/kilo wt. orally	20.5	25.3	2425	4
Group C Mitomycin C total dose 14 mg/kilo wt. and 2-DG, total dose 5.6 gm/kilo wt.	21.6	20.8	234*	12
Group D control no treatment	21.6	27.4	3250	4

* In 20 per cent of mice, there was complete disappearance of growth.

of all three types of transplanted tumors was strikingly increased. The final average tumor weight, after fourteen days of therapy was for sarcoma 180—234 mgs.; for Ehrlich carcinoma—167 mgs., and for Crockers rat carcinoma—453 mgs., with the tumors in 10 to 24 per cent of animals disappearing completely.

The toxic manifestations, such as weight loss was slightly more pronounced in the groups of animals receiving the combined therapy than in other treated groups, but the mortality rate was insignificantly small.

The results so obtained stress the additive inhibitory influence of 2-DG in the Mitomycin C therapy.

D-Gluco-ascorbic and Dehydroascorbic acids

Robertson *et al.* (39) reported that a scorbutigenic regime greatly inhibited the growth of fibrosarcoma in guinea pigs. Sokoloff *et al.* (54, 58) confirmed this observation by finding that a vitamin C-free diet resulted in a decrease in the total and nuclear DNA contents in guinea pigs fibrosarcoma A1-2, and that a similar inhibitory effect, although of a lesser degree, was observed upon administration of d-glucoascorbic acid, to rats bearing the Crocker rat carcinoma. Miller *et al.* (33) and Miller and Sokoloff (34) disclosed that low ascorbic acid concentrations in the blood of animals and man increased the

TABLE II

Additive Inhibitory Effect of 2-Deoxy-d-Glucose and Mitomycin C on Ehrlich Mouse Carcinoma

48 hour transplant

Mitomycin C, total dose 14 mg/kilo wt./mouse, given intraperitoneally in fourteen days. 2-DG, total dose 5.6 gm/kilo wt./mouse, orally, in fourteen days.

Group A.—Mitomycin C alone
Group B.—2-DG, alone
Group C.—Mitomycin C and 2-DG
Group D.—Control, no treatment

Each group of 25 mice

Groups	Initial av. wt. gm.	Final av. wt. gms. after 15 days	Tumor av. wt. mgs. after 15 days	Mortality Rate %
Group A Mitomycin C total dose 14 mg/kilo wt. intraper.	20.2	21.7	758	0
Group B 2-DG total dose 5.6 gm/kilo wt. orally	21.6	26.5	1980	0
Group C Mitomycin C total dose 14 mg/kilo wt. and 2-DG total dose 5.6 gm/kilo wt.	22.4	20.6	167*	15
Group D control no treatment	21.9	27.9	2965	4

* In 24 per cent of mice, there was complete disappearance of growth.

TABLE III

ADDITIVE INHIBITORY EFFECT OF 2-DEOXY-D-GLUCOSE AND MITOMYCIN C ON CROCKER RAT CARCINOMA

48 hour transplant

Mitomycin C, total dose 8.0 mg/kilo wt./rat, intraperitoneally in fourteen days.
2-DG, total dose 4.2 gm/kilo wt., orally, in fourteen days.

Group A.—Mitomycin C alone
Group B.—2-DG alone
Group C.—Mitomycin C and 2-DG
Group D.—Control, no treatment

Each group of 20 rats

Groups	Initial av. wt. gm.	Final av. wt. gms. after 15 days	Tumor av. wt. mgs. after 15 days	Mortality rate %
Group A Mitomycin C total dose 8.0 mg/kilo wt. intraper.	110.2	124.1	1124	0
Group B 2-DG total dose 4.2 gm/kilo wt. orally	109.5	128.8	4856	5
Group C Mitomycin C total dose 8.0 mg/kilo wt. and 2-DG total dose 4.2 gm/kilo wt.	112.3	118.5	453*	10
Group D Control no treatment	108.6	146.4	7892	10

* In 10% of rats, tumor disappeared completely.

radiosensitivity of malignant tissue. Wooley and Krampitz (81) and Woolley (80, 82), postulated that d-glucoascorbic acid is an analog of ascorbic acid. Banerjee and Elvehjem (2), Gorlin (22) Zilva (83) and others were unable to find any evidence of scurvy induced by D-GA. However, Sokoloff *et al.* (55, 56, 57, 58) and others demonstrated that D-GA decreased blood ascorbic acid content in rats, and when added to a Sherman-LaMer ration in 0.75 per cent, it moderately inhibited Crocker rat carcinoma and mouse adenocarcinoma E 0771, but did not influence the incidence of lymphatic

leukemia in the Ak-1m strain of mice, prolonging, however, the life span of leukemic mice by 26.8 per cent.

Goldstein *et al.* (21) revealed that a scorbutigenic diet induced changes in the PNA and DNA content of the spleen and adrenals of guinea pigs and increased the PNA/DNA ratio. On the basis of his experiments *in vitro,* they concluded that ascorbic acid is involved in the formation of DNA from PNA. According to Nichol and Welch (36), Broquist *et al.* (5), Hill and Scott (26), and Welch *et al.* (76), ascorbic acid, a reducing agent, enhances the formation of citrovorum factor (CFO) from folic acid (PGA). Thus, decrease in the ascorbic acid content of blood and tissue might affect the formation of folic acid, and thus influence indirectly the growth processes.

On the other hand, Heise (24, 25) reported that dehyroascorbic acid administered to Jensen rat sarcoma or Ehrlich mouse carcinoma, in a dose of from 2.0 up to 200 mg/kilo wt. inhibited considerably tumor growth. The smaller doses (2.0 mg/kilo wt.) were more effective than the larger ones. According to this author, tumor slices of Jensen sarcoma or ascites tumors were found to increase up to 64 per cent in respiration and decrease in 33 per cent in glycolysis upon exposure to dehydroascorbic acid. This effect seems to be related to that produced by oxidized glutathione. The oncostatic activity of dehydroascorbic acid in the experiments of Heise, might have been caused by an isomer of it, for L-dehydroascorbic acid is actually a major intermediate in the metabolic pathway from L-ascorbic acid toward CO_2, oxalate, L-xylose, glucose and glycogen as indicated by the work of Chan *et al.* (9) and others. For this reason, both D-GA and L-DA were investigated by us on their inhibitory influence on transplanted tumors, alone and in combination with Mitomycin C.

Experimental. In this series of tests, the following procedure was applied both for Ehrlich mouse carcinoma and Crocker rat carcinoma. Mice and rats were placed on the Sherman-LaMer scorbutigenic diet, five days prior to tumor transplantation. This diet was composed of: 18 gm. vitamin-free casein; 65 gm. corn starch; 5 gm. vegetable oil (Mazola); 2 mg. cod liver oil; 6 gm. dried brewers yeast; and 4 gm. salt mixture #1. Each trial, either with Ehrlich carcinoma or Crocker rat carcinoma, consisted of 120 animals, divided into six groups of twenty animals each. Group A: control; Group B: 2 per cent of D-GA was added to the ration; Group C: 2 per cent of D-GA in

the ration and Mitomycin C, total dose 14 mg/kilo wt./mouse or 8.0 mg/kilo wt./rat administered intermittently; Group D: 2 per cent of L-DA was added to the ration; Group E: 2 per cent L-DA in the ration and Mitomycin C, total dose 14 mg/kilo wt./mouse or 8.0 mg/kilo wt./rat, administered intermittently. The last Group F, received only Mitomycin C, the same total dose as in Groups C and E. The results of these trials are summarized in Tables IV and V.

TABLE IV

EFFECT OF MITOMYCIN C COMBINED EITHER WITH D-GLUCO-ASCORBIC ACID OR L-DEHYDROASCORBIC ACID ON EHRLICH MOUSE CARCINOMA

48 hour transplant

Mitomycin C, total dose 14/mg kilo wt./mouse, intermittently in fourteen days.
D-GA, 2 per cent added to Sherman-LaMer diet for 21 days.
L-DA, 2 per cent added to Sherman-LaMer diet for 21 days.

Each group 20 mice

Group A.—Control, no treatment
Group B.—D-GA alone
Group C.—D-GA and Mitomycin C
Group D.—L-DA alone
Group E.—L-DA and Mitomycin C
Group F.—Mitomycin C alone

Groups	Initial av. wt. gm.	Final av. wt. after 15 days gm.	Tumor av. wt. after 15 days mg.	Mortality Rate %
Group A Control	19.8	26.5	3096	5
Group B D-GA, 2% in ration	20.1	24.1	2240	0
Group C D-GA, 2% in ration Mitomycin C intrap.	20.2	21.2	560	10
Group D L-DA, 2% in ration	21.5	27.6	3424	5
Group E L-DA, 2% in ration, Mitomycin C intrap.	20.4	22.4	1125	0
Group F Mitomycin C intrap.	20.2	22.2	810	0

TABLE V

EFFECT OF MITOMYCIN C COMBINED EITHER WITH D-GLUCO-ASCORBIC ACID OR L-DEHYDROASCORBIC ACID ON CROCKER RAT CARCINOMA

48 hour transplant

Mitomycin C, total dose 8.0 mg/kilo wt./rat, intermittently in fourteen days.
D-GA, 2 per cent added to Sherman-LaMer diet for 21 days.
L-DA, 2 per cent added to Sherman-LaMer diet for 21 days.

Each group 20 rats

Group A.—Control, no treatment
Group B.—D-GA alone
Group C.—D-GA and Mitomycin C
Group D.—L-DA alone
Group E.—L-DA and Mitomycin C
Group F.—Mitomycin C alone

Groups	Initial av. wt. gm.	Final av. wt. after 15 days gm.	Tumor av. wt. after 15 days mg.	Mortality Rate %
Group A Control	112.5	149.2	8325	5
Group B D-GA, 2% in ration	110.2	136.8	6150	10
Group C D-GA, 2% in ration, Mitomycin C intrap.	106.5	121.4	720	10
Group D L-DA, 2% in ration	112.1	144.3	8885	0
Group E L-DA, 2% in ration, Mitomycin C intrap.	111.7	128.7	2686	5
Group F Mitomycin C intrap.	108.8	122.4	1085	10

Analysis of Results. The figures of Tables IV and V offer some interesting data. When D-GA was added in 2 per cent to the Sherman-LaMer diet, both Ehrlich mouse carcinoma and Crocker rat carcinoma showed moderate inhibition as compared with control. In the case of Crocker carcinoma, the average tumor weight of rats, kept for twenty-one days on the diet with D-GA was 6150 mgs.

against 8325 mgs., for control, or an inhibition index of about 25 per cent.

With Ehrlich carcinoma, the average tumor weight in mice on the diet with D-GA for three weeks was 2240 mgs. against 3096 mgs. for control, or an inhibition of about 30 per cent. When a combined treatment with Mitomycin C and D-GA was applied, there was a definite but moderate synergestic action. The average Ehrlich tumor weight was 560 mgs. against 810 mgs. for Mitomycin C alone; and the average Crocker tumor weight was 720 mgs. against 1085 mgs. for Mitomycin C alone. In neither groups was there a complete regression of tumors.

Quite different results were obtained with L-dehydroascorbic acid. The average Crocker tumor weight after the rats were kept for three weeks on the diet with 2 per cent of L-DA, was 8885 mgs. against 8325 mgs. for control, or a gain of an average 560 mgs. Similar data were obtained with Ehrlich mouse carcinoma. On the L-DA diet, the average tumor weight was 3424 against 3096 mgs. for control, or about a 10 per cent gain in tumor weight.

The combined treatment with Mitomycin C and L-DA gave no indication of any synergestic action of the two substances. In fact, the oncolytic activity of Mitomycin C was less pronounced in animals kept on the diet with L-DA, than in mice and rats on a ration without L-DA. All in all, one may state that L-dehydroascorbic acid added in 2 per cent to the Sherman-LaMer diet slightly enhanced growth of Ehrlich mouse carcinoma and Crocker rat carcinoma in animals, either treated or not-treated with Mitomycin C.

8-Azaguanine and 6-Mercaptopurine

During the last several years, many attempts were made to apply a multiple chemotherapy of oncostatic substances to experimental tumors, or even in the clinical treatment of cancer. While a synergestic action of oncostatic agents on solid transplanted tumors was reported by Sartorelli and LePage (40), Tarnowski and Stock (73), Clarke *et al.* (12, 13), Sols and Crane (66), Fernandes *et al.* (20), Shapiro *et al.* (43, 44, 45), and others, the application of multiple therapy of oncostatic agents in the treatment of human cancer was rather disappointing, according to Shapiro and Fugmann (46), Clarke *et al.* (14), Steinfeld *et al.* (67) and others.

In the present study, two antimetabolites, 8-Azaguanine and 6-

Mercaptopurine were investigated alone and in combination with Mitomycin C, on their possible synergestic influence upon solid tumors, mouse sarcoma 180, Ehrlich mouse carcinoma and Crocker rat carcinoma.

8-Azaguanine. Kidder *et al.* (27, 28) were the first to report in 1949 that 8-Azaguanine, the analog of guanine, exerted a strong oncostatic influence on mouse adenocarcinoma E 0771. Sokoloff *et al.* (53) confirmed their findings on Crocker rat carcinoma but pointed out that the analog was much less effective when the transplanted tumor was in an advanced stage. Carlo and Mandel (7, 8) and Mandel and Law (32) reported that a pronounced potentiation of the carcinostatic effect of 8-azaguanine was produced by an additional administration of 4-amino-5-imidazolecarboxamide. Shapiro and Fugmann (43) and Woodside and Kelton (78) obtained moderate potentiation of the oncostatic activity of 8-azaguanine by combining it with flavotin, analog of riboflavin. Shapiro *et al.* (45) disclosed that the three-compound combination of 8-azaguanine, nicotinamide and testosterone caused cessation of 755 tumor growth.

Experimental. Both 8-azaguanine and Mitomycin C were administered in subtoxic doses. Mitomycin C, total dose 14.0 mg/kilo wt./mouse and 8 mg/kilo wt./rat, was inoculated intraperitoneally, in fourteen days, intermittently, every second day. 8-azaguanine, total dose, 112 mg/kilo wt./mouse and 84 mg/kilo wt./rat was given subcutaneously on fourteen consecutive days. This combined treatment was tested on mouse sarcoma 180, Ehrlich mouse carcinoma and Crocker rat carcinoma. The results of these tests are summarized in Table VI.

Analysis of Results. The data presented in Table VI indicates that the oncolytic activity of Mitomycin C was potentiated by additional administration of 8-azaguanine, without any significant increase in toxicity of the combined treatment. 8-azaguanine, total dose 112 mg/kilo wt./mouse, administered consecutively in fourteen days to mouse bearers of Sarcoma 180 or Ehrlich carcinoma inhibited by itself these two solid tumors by about 40–50 per cent. When combined with subtoxic doses of Mitomycin C, inhibition of the growth was considerably increased, with a number of tumors regressed completely. After fourteen days of combined therapy, the average weight of Ehrlich carcinoma was about thirteen times smaller than that of the control tumor, and in the case of Sarcoma 180, the inhibition

TABLE VI

ADDITIVE EFFECT OF 8-AZAGUANINE AND MITOMYCIN C ON SARCOMA 180, EHRLICH CARCINOMA AND CROCKER RAT CARCINOMA

48 hour transplant

Mitomycin C, total dose 14 mg/kilo wt./mouse and 8 mg/kilo wt./rat, intermittently, in fourteen days.

8-Azaguanine, total dose 112 mg/kilo wt./mouse and 84 mg/kilo wt./rat consecutively in fourteen days.

Each group 20 animals

Groups	Initial av. wt. gm.	Final av. wt. gm. after 15 days	Tumor av. wt. mg. after 15 days	Mortality Rate %
		Ehrlich carcinoma		
Group A Control	22.4	28.5	2895	5
Group B 8-azaguanine, alone	21.3	26.7	1450	0
Group C Mitomycin C, alone	20.8	22.9	815	5
Group D 8-azaguanine & Mitomycin C	22.5	23.2	212*	10
		Sarcoma 180		
Group A Control	19.8	26.6	2565	5
Group B 8-azaguanine, alone	20.8	25.4	1520	0
Group C Mitomycin C, alone	22.9	23.8	895	0
Group D 8-azaguanine & Mitomycin C	21.8	23.1	386†	10
		Crocker rat carcinoma		
Group A Control	108.5	142.2	8340	5
Group B 8-azaguanine, alone	109.8	138.8	4560	5
Group C Mitomycin C, alone	111.2	123.7	1058	10
Group D 8-azaguanine & Mitomycin C	108.8	122.3	582‡	10

* 20% complete regression
† 15% complete regression
‡ 10% complete regression

was somewhat smaller, with the average tumor weight reduced to about one-seventh of control. The combined treatment of Crocker rat carcinoma showed a similar trend. The average tumor weight in the treated group was about fifteen times smaller than those of the control. Apparently there was some synergestic action of the two substances, one of which is oncostatic and the other oncolytic, as far as the three transplanted tumors were concerned.

6-Mercaptopurine. Elion *et al.* (16, 17, 18) synthesized 6-Mercaptopurine in 1952 and found it to be a purine antagonist in *Lactobacillus casei.* They revealed that this antimetabolite prevented utilization of hypoxanthine primarily, and of adenine secondarily. Clarke *et al.* (10, 11) reported that 6-mercaptopurine was an inhibitor of Sarcoma 180. Although the inhibition rate induced on Sarcoma 180 by 6-mercaptopurine was less pronounced than that of 4-amino-folic acid or triethylene melanine, the oncostatic effect of this agent was more persistent. This inhibition was reversed by any of the four purine bases, according to Hamilton *et al.* (23). Ultmann and Feigelson (74) reported that 6-MP inhibited xanthine catabolism *in vivo* in rat, being involved in the xanthine oxidase system. Skipper *et al.* (52) reported that 6-MP was a moderate inhibitor of sarcoma 180, but adenocarcinoma 755 was profoundly arrested by the purine antagonist. On the other hand, thioguanine (2-amino-6-mercaptopurine) was found to be about 20-fold as active, as well as toxic, as 6-MP, according to Clarke *et al.* (14). In view of the high toxicity of thioguanine, in our present study we limited our tests to 6-MP, alone and in combination with Mitomycin C.

Experimental. Both agents, 6-MP and Mitomycin C were administered to mice bearing Sarcoma 180 and Ehrlich carcinoma and rats, bearers of Crocker carcinoma, in subtoxic doses.

The total dose of Mitomycin C was 14 mg./kilo wt./mouse and 8.0 mg/kilo wt./rat given intermittently in fourteen days. The total dose of 6-Mercaptopurine was 280 mg/kilo wt./mouse and 210 mg/kilo wt./rat, administered in fourteen days, every second day, seven intraperitoneal injections altogether. Table VII presents the summary of the results of these tests.

Analysis of Results. The figures of Table VII indicate that the combined treatment of Mitomycin C and 6-MP, both administered in subtoxic doses, was very toxic to mice and rats. At the total dose of Mitomycin C, 14 mg/kilo wt./mouse and 6-MP, 280 mg/kilo

wt./mouse, given in fourteen days, 40–45 per cent of the mice died, some of them on the eighth and tenth days of treatment. There was considerable weight loss in the two groups of mice, with Sarcoma 180

TABLE VII

ADDITIVE EFFECT OF 6-MERCAPTOPURINE AND MITOMYCIN C ON SARCOMA 180, EHRLICH CARCINOMA AND CROCKER RAT CARCINOMA

48 hour transplant

Mitomycin C, total dose 14 mg/kilo wt./mouse and 8 mg/kilo wt./rat, intermittently, in fourteen days.

6-Mercaptopurine, total dose 280 mg/kilo wt./mouse and 210 mg/kilo wt./rat, in fourteen days, intermittently.

Each group 20 animals

Groups	Initial av. wt. gm.	Final av. wt. gm. after 15 days	Tumor av. wt. after 15 days mg.	Mortality Rate %
		Ehrlich carcinoma		
Group A Control	20.2	27.5	2654	5
Group B 6-MP, alone	19.8	24.3	1768	10
Group C Mitomycin C, alone	20.1	21.3	866	10
Group D 6-MP and Mitomycin C	22.2	18.5	220*	45
		Sarcoma 180		
Group A Control	21.3	28.4	3210	0
Group B 6-MP, alone	21.4	26.5	2605	5
Group C Mitomycin C, alone	21.5	23.0	834	10
Group D 6-MP and Mitomycin C	21.0	18.4	280*	40
		Crocker rat carcinoma		
Group A Control	120.4	158.3	8896	5
Group B 6-MP, alone	118.5	148.2	6585	10
Group C Mitomycin C, alone	121.1	132.2	1104	5
Group D 6-MP and Mitomycin C	118.6	120.5	357*	30

* The dead animals were not included in calculating the average tumor weight.

and Ehrlich carcinoma. The same picture was in evidence in regard to Crocker rat carcinoma. When a combined total dose, Mitomycin C, 8 mg. and 6-MP 210 mg/kilo wt./rat was administered to rats in fourteen days, 30 per cent of the animals died before the treatment was completed. The weight loss in rats was also apparent, although less pronounced than in the mice. In another series of trials, the total dose of 6-MP was reduced by two: 140 mg/kilo wt./mouse and 105 mg/kilo wt./rat. This dose by itself is only slightly toxic to mice and rats. Yet when combined with Mitomycin C, it caused a mortality rate of 20–25 per cent. Under these circumstances, the decrease in average tumor weight in the groups of animals receiving the combined treatment was of little, if any, significance.

Discussion

The intrinsic nature of the oncolytic action of Mitomycin C is not defined as yet. The study of Sekiguchi and Takagi (42) and Shiba *et al.* (47) pointed to the selective inhibition of the biosynthesis of deoxyribonucleic acid. Reilly *et al.* (38) believed in the interference by the antibiotic with *de novo* synthesis of purines, prior to the formation of the purine precursor, 5-amino-4-imidazole carboxamine. Phillips *et al.* (37) admitted the possibility of either type of interference with the synthesis of purines, without giving any new data in this respect. The fact remains, however, that so far any attempt to show *in vitro* any inhibition of either PNA, DNA or protein synthesis gave negative results (4). Our cytopathologic study on Mitomycin C, as reported in our previous paper (64) seems to indicate that the mode of action of Mitomycin C is very similar to that of Myleran or nitrogen mustard. It is from this view point that the combined treatment with the antimetabolite of glucose, 2-Deoxy-D-glucose, acquires particular interest.

The work of Bernstein (3), Low (31), Schmitz *et al.* (41) and others indicates that the pentoses of PNA and DNA are formed from glucose metabolites by enzymic action. Schmitz and his coworkers demonstrated in their studies on alternative pathways of glucose metabolism in tumor tissues *in vivo* that both PNA and DNA of the tumors incorporated considerable amounts of radio-active glucose, thus suggesting the potential significance of antimetabolites of glucose in our search for tumor inhibitors. The results of combined

treatment of transplanted tumors by Mitomycin C and 2-DG, as reported in this paper, seem to indicate rationality of this approach.

The attempt to combine Mitomycin C with 8-azaguanine and 6-mercaptopurine, both the antimetabolites interfering with *de novo* synthesis of purine, revealed moderate synergestic action with a relatively non-toxic agent, 8-azaguanine. With 6-MP, the results were obscured by a high toxicity of the combined treatment. Thus in this case we were confronted with an accumlative toxic effect, in spite of the fact that the two compounds were taken in subtoxic doses. We met here with the same phenomenon as the one we observed when various combined therapies were used by us on ascites tumors. The toxicity of an oncolytic or an oncostatic agents remains, so far, the paramount obstacle in almost all attempts to combine them in the treatment of human cancer.

Summary

Mitomycin C, in subtoxic total dose, was administered, intermittently, in combination with 2-Deoxy-D-glucose, D-glucoascorbic acid, 8-Azaguanine, and 6-Mercaptopurine to mice, bearers of solid Ehrlich carcinoma and sarcoma 180, and to rats, bearers of Crocker carcinoma.

The analog of glucose, 2-DG, considerably potentiated the oncolytic activity of Mitomycin C, while the analog of ascorbic acid, only moderately increased its inhibitory influence.

The analog of guanine, 8-Azaguanine, when combined with Mitomycin C, exerted certain synergestic action in the transplanted tumors.

There was an accumulative toxic effect when Mitomycin C was combined with 6-Mercaptopurine, resulting in a high mortality rate among the treated animals.

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A COMPARATIVE STUDY OF GROWTH AND DEVELOPMENT OF THE KANGAROO RATS, *DIPODOMYS DESERTI* STEPHENS AND *DIPODOMYS MERRIAMI* MEARNS

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During a recent study of sexual behavior and reproduction of the kangaroo rats, *Dipodomys deserti* and *Dipodomys merriami*, a comparison of growth and development of closely related sympatric species reared under identical laboratory conditions was possible. Litters born in the laboratory were carefully examined and measured from birth to maturity.

Although growth in other genera of mammals has been carefully studied, few instances of breeding in the genus *Dipodomys* have been recorded and limited growth data are available. The solitary nature of the animals prevents laboratory breeding under ordinary conditions. Animals confined in restricted space engage in fighting which usually results in the death of one or both of them. Chew and Butterworth (3) published an analysis of growth and development of Merriam's kangaroo rat, *D. merriami* in which the senior author was successful in laboratory breedings of this species. The scattered literature on growth and development in the genus *Dipodomys* is summarized in their paper.

MATERIALS AND METHODS

A total of 32 laboratory animals representing 8 *D. deserti* litters and 4 *D. merriami* litters that were the direct progeny of wild parents were used in this study. Seven of the 8 litters of *D. deserti* were the products of laboratory breedings, the first recorded instances for this particular species. The other litters were from pregnant females captured in the field and brought back to the laboratory for observation and which subsequently produced young.

The parent animals were all obtained from one locality at the western edge of the Mojave Desert near the base of Alpine Butte located approximately 16 miles northeast of Palmdale in Los Angeles

County, California. The altitude of the collecting area was approximately 1000 meters (3261 feet).

The captive animals were placed in two large breeding cages measuring 3 by 4 meters at the University of Southern California. A partition separated the two cages and sand and desert soil up to several inches in depth was spread on the floor. Animals were allowed to run in these cages without restriction. Nesting sites consisting of glass bottles, empty cardboard mailing tubes and cardboard boxes were provided. One pair of each species was placed in each cage. Instances of breeding in *D. deserti* occurred in these cages. Animals were fed rolled oats and sunflower seeds. Lettuce was provided and water was also made available. An excess amount of food material was always available.

All animals were measured from birth and the measurements were continued daily or at frequent intervals until adult sizes were attained. Standard measurements of total length, tail length, hind foot length, ear (from the notch) length, and body weight were taken. Measurements were analyzed as in Brody (2) and values were plotted on a semilogarithmic scale against age on the arithmetic scale. Linear segments of such a plot indicate periods when growth increments are constant percentages of previous sizes. From these linear sections instantaneous growth rates were calculated as:

$$K = \frac{\ln m_2 - \ln m_1}{t_2 - t_1}$$

The value of K is the instantaneous percentage rate of growth for the unit of time in which t_2 and t_1 are expressed. Ln m_2 and ln m_1 are natural logarithms of the measurements made at t_1 and t_2.

Observations and Discussion

General Development. The general pattern of development in *D. deserti* is similar to that described by Chew and Butterworth (3) for *D. merriami.* The desert kangaroo rat is born hairless and has a thin, pink, wrinkled, transparent integument. Viscera are apparent through the skin of the venter and sutures and blood vessels on the skull are visible. The snout area containing the vibrissa sheaths appear to be swollen and are richly vascular. The yellowish-brown vibrissae are about 6 millimeters long at birth, but lengthen to 13 mm. in 5 days and

23 mm. by 15 days. The adult length of 72 mm. is attained by 90 days.

Black pigmentation appears about 5 days after birth. The pigmentation begins on the dorsum and top of the head. A faint dorsal tail stripe is present. The end of the tail is unpigmented for 10 mm. and then a black ring circles the entire tail for about 7 mm. The black area extends on the dorsal side of the tail for about 30 mm. and shades out to a pale gray color. The entire ventral surface of the tail is white except for the small black area near the tip. The feet and venter are white. The site of the dermal gland shows as a light depression just posterior to the scapulae. It darkens by 15 days, begins to lighten by 19 days and is covered with hair by 21 days. The head is sufficiently pigmented by 7 days so that the dorsal cranial sutures and blood vessels are no longer visible. By 21 days the mammae have become very distinct. The insides of the thighs and the venter, in general, are sparsely haired until about 15 days. By 21 days the animal is fully furred and the color pattern of the young is now similar to that of the adult. The young appear darker than the parents, however.

The pelage of *D. merriami* is a darker yellowish-buff than that of *D. deserti,* the white tail stripe is wider than the dark tail stripe, and the terminal tuft is brown. Dark whisker patches are distinct in *D. merriami* but are absent in *D. deserti.* Juvenile *D. merriami* are completely furred by 15 days. Developmental stages are shown in Figures 1 to 6.

The pinnae of the ears, closed at birth, and only two mm. in length gradually open from 9 to 15 days. The length of time until opening varies with different litters. The ears are fully opened by 15 days in *D. deserti* and by 10 days in *D. merriami.*

The nails are soft at birth and gradually become hard by 15 days in both species.

The incisor teeth appear later in *D. deserti* than in *D. merriami.* They break through the gums at about 2 days in the latter (Chew and Butterworth, 3) while they do not appear until about 9 days in the former. Teeth are white at first but gradually darken to yellow. By 25 days the teeth of *D. deserti* are strong enough to pierce the skin of man.

Eye development is summarized in Figure 7 for various species of kangaroo rats. In my laboratory, individuals of *D. deserti* had their eyes open as follows: 3 on the 15th day; 3 on the 16th day and 6 on

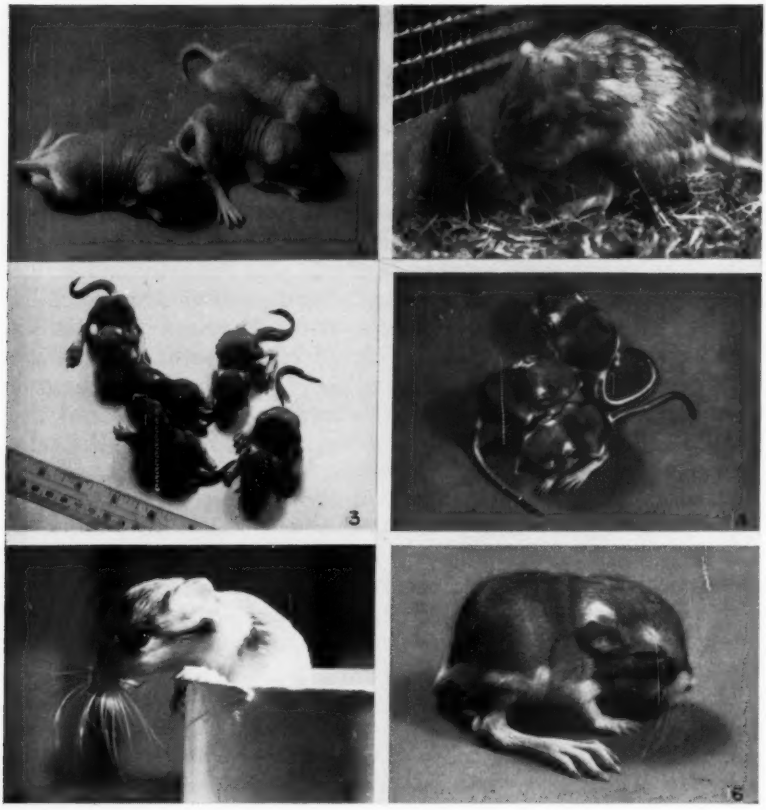

FIGURE 1

Dipodomys deserti, 3 days old. Note absence of hair and the relative sizes of the feet. Both eyes and ears are tightly closed.

FIGURE 2

Dipodomys merriami, a mother nursing her young.

FIGURE 3

Dipodomys deserti, 10 days old. A litter of 5. Note the white tail tip, one of the distinguishing features of this species.

FIGURE 4

Dipodomys merriami, 11 days old. A litter of 3.

FIGURE 5

Dipodomys deserti, 16 days old. From the same litter as pictured in Figure 3.

FIGURE 6

Dipodomys merriami, 16 days old

the 17th day. *D. merriami* had their eyes open between the 11th and 15th days.

Species	Days after birth that eyes open	Reference
Dipodomys deserti	15–17	Butterworth
D. deserti	16	Rush (8)
D. merriami	11	Butterworth
D. merriami	11–15	Chew and Butterworth (3)
D. merriami	21	Doran (5)
D. merriami	by third week	Reynolds (7)
D. heermanni	12–15	Fitch (6)
D. heermanni	14–16	Tappe (9)
D. nitratoides	13–14	Culbertson (4)
D. spectabilis	14	Bailey (1)

FIGURE 7
Eye development in various species of kangaroo rats.

A comparison of general development in the two species in this study is shown in Figure 8.

	Days after birth	
	D. deserti	*D. merriami*
Eyes open	11–17	11–15
Ears open	9–15	8–10
Incisor teeth erupt	7–10	2–8
Solid food eaten	15	13
Solid feces first noted	15	17
Nails harden	12–15	12–15
Sand used for cleaning pelage	17	13–15
Well haired	11–15	14
Weaned	15–25	17–22
Mammae first visible	21	11
Drumming with feet first noted	33	—

FIGURE 8
Comparison of sequences of general development of *Dipodomys deserti* and *Dipodomys merriami*. Selected features based on 8 litters of *D. deserti* and 4 litters of *D. merriami*.

Growth Analyses: A comparison of weight increases in *D. deserti* and *D. merriami* is shown in Figures 9 and 10. Weights become constant at adult levels of approximately 145 grams in *D. deserti* and 40 grams in *D. merriami*. An analysis indicates that early growth is rapid in *D. merriami* and continues more slowly toward the maximum weight. At 10 days *D. deserti* had reached 16 per cent of its total adult weight while *D. merriami* had attained 26 per cent of its adult weight. At 30 days the two species had reached approximately half their maximum weight, 47 per cent in *D. deserti* and 53 per cent in *D. merriami*. At

	15 days		20 days		30 days		50 days		90 days	
	D.d.	D.m.	D.d.	D.m.	D.d.	D.m.	D.d.	D.m.	D.d.	D.m.
Total length	47	54	55	70	72	80	83	95	97	99
Tail length	39	47	50	61	73	81	91	97	93	99
Foot length	70	82	80	87	82	95	96	99	100	100
Ear length	63	63	67	82	78	90	81	95	88	99
Weight	27	35	33	43	47	53	75	64	91	78

FIGURE 9

Percentages of growth toward maturity completed at indicated intervals in *D. deserti* and *D. merriami*.

50 days *D. deserti* had reached 75 per cent of its adult weight while *D. merriami* had only attained 64 per cent. At this point *D. merriami* lagged behind and then very slowly approached the maximum weight. At 90 days *D. deserti* had attained 91 per cent of its weight while in *D. merriami* only 78 per cent had been reached. In the later phases of growth *D. merriami* gained weight more slowly than *D. deserti*. Both attained full adult average weights by 150 to 180 days.

In total length (Figures 9 and 11) *D. merriami* grew more rapidly than *D. deserti* throughout the growth period. Maximum adult lengths are difficult to measure but approximate adult dimensions are attained by 90 days.

The tail of *D. merriami* (Figures 9 and 11) grew faster than that of *D. deserti* during the developmental period. After 90 days *D. merriami* had little tail growth while *D. deserti* continued to increase its tail length by 7 per cent.

The foot (Figures 9 and 12), which was already well developed at birth, grew fastest of all. At 15 days *D. deserti* had a foot 70 per cent of adult size while *D. merriami* had attained 82 per cent of the adult foot size. Both species attained maximum foot size between 50 and 90 days.

The ear (Figures 9 and 12) grew rapidly in both species, that of *D. merriami* faster than that of *D. deserti*. The ear was approximately fully grown by 90 days in *D. merriami* but continued to grow slowly in *D. deserti*.

Size of the litter (Figure 13) made little difference on increases in weight of *D. deserti*. Litters containing 2, 3, 4, and 5 individuals were compared and showed very little variation in weight changes. Apparently all individuals in the respective litters received sufficient food both by nursing and individually after weaning. Litters of *D. merriami* were not numerous enough to permit these observations.

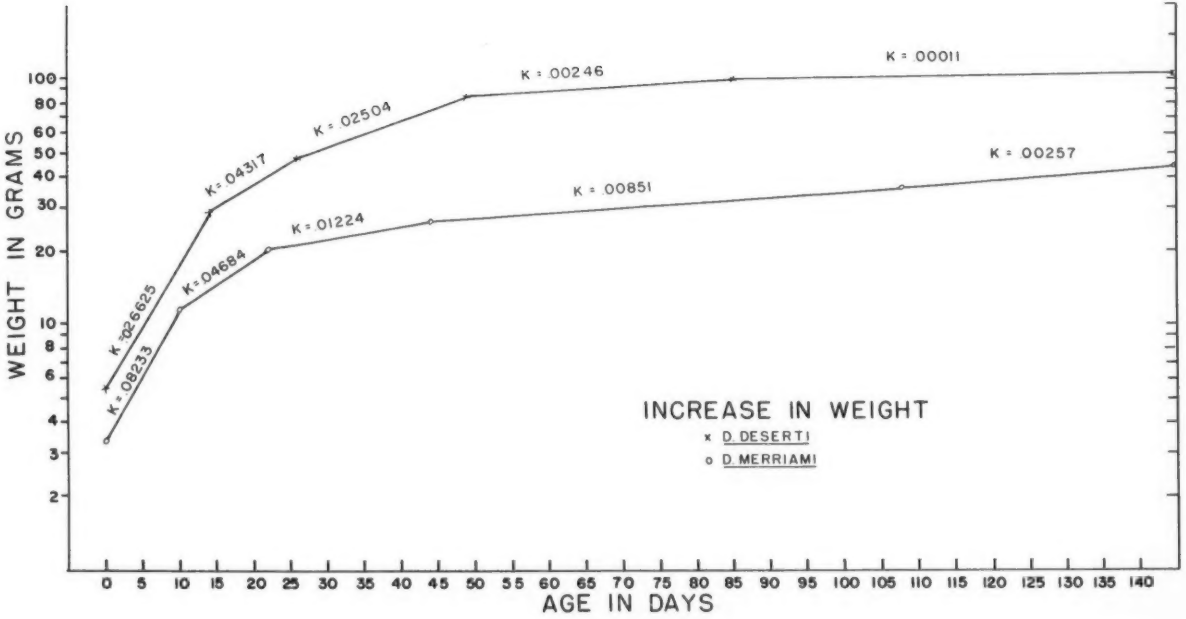

FIGURE 10

A comparison of weight increases during the growth of *D. deserti* and *D. merriami*, semilogarithmic plot; the value times 100 equals the percentage of increase per day during a particular growth phase. The *K* values above the segments of the plot are instantaneous growth rates. Weights are means of 12 litters of animals.

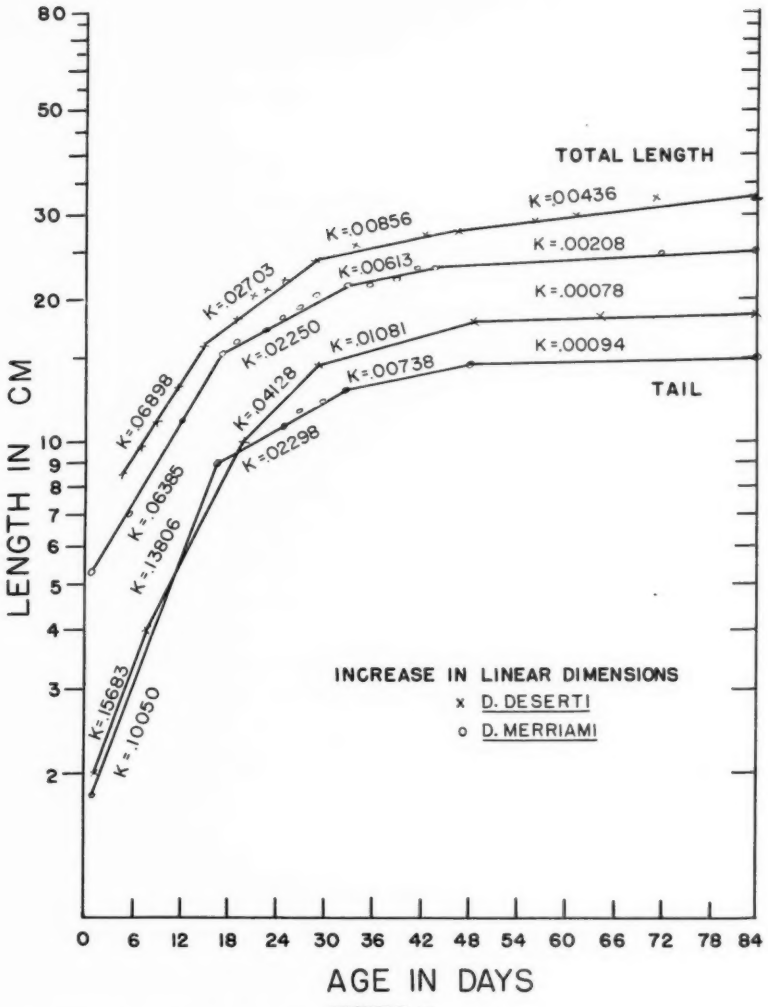

FIGURE 11

A composite graph showing increase in linear dimensions of total length and tail length in *D. deserti* and *D. merriami,* semilogarithmic plot.

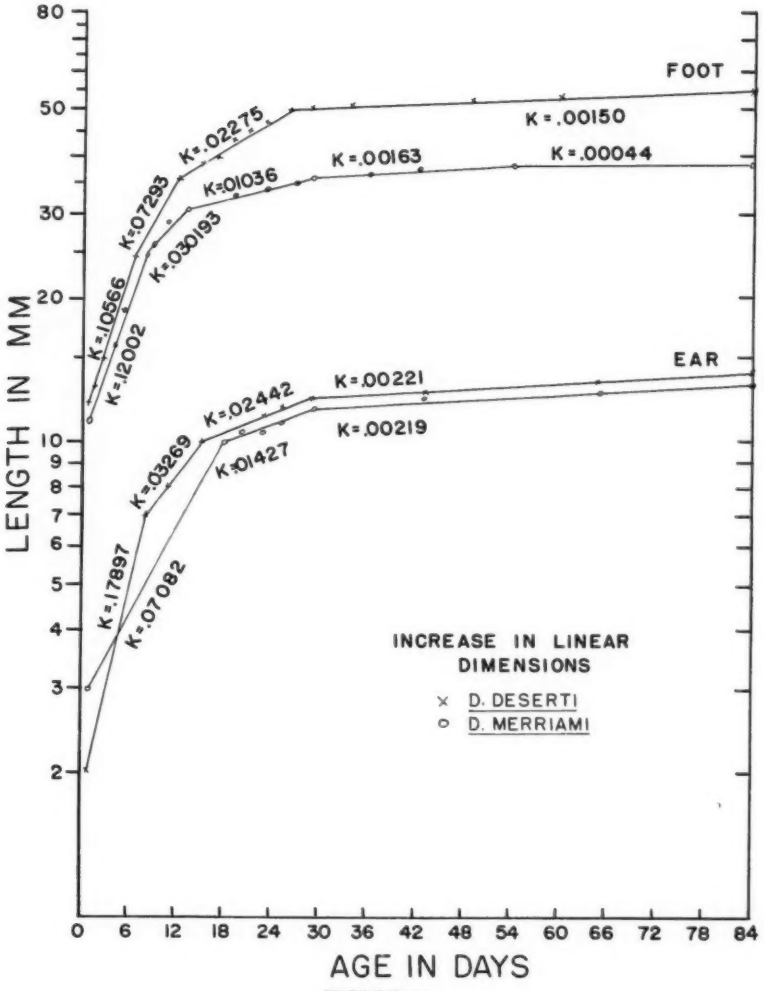

FIGURE 12

A composite graph showing increase in linear dimensions of foot length and ear length of *D. deserti* and *D. merriami,* semilogarithmic plot.

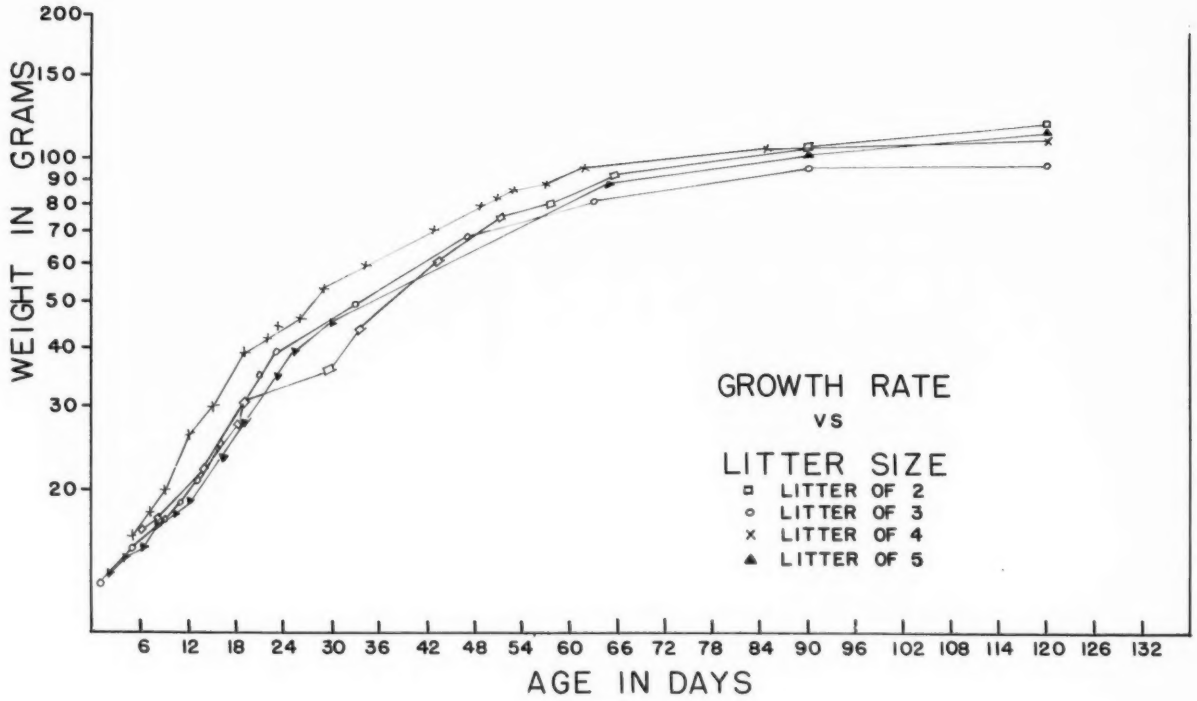

FIGURE 13
Growth rate versus litter size in *D. deserti* semilogarithmic plot. Measurements are means for each litter.

The differential rates of growth during development in the two species of kangaroo rats is significant. The smaller *D. merriami* matures more rapidly than the larger *D. deserti*. The development of various body parts in growing juveniles of *D. merriami* demonstrates more rapid growth and an earlier maturity than *D. deserti*. The precocious growth, especially during early development, brings *D. merriami* to an earlier seasonal reproductive potential than *D. deserti* and may allow for an earlier dispersion. Rapid development may be one contributing factor for the larger geographical range of Merriam's kangaroo rat.

Summary and Conclusions

The growth and development of two sympatric species of kangaroo rats is discussed, utilizing data from 8 litters of *D. deserti* and 4 litters of *D. merriami* reared under identical laboratory conditions. General development and sequences of hair acquisition are described. Animals of both species were completely furred by 15 days. Their pelages were darker than those of their parents.

The ears, which were closed at birth, opened in 15 days in *D. deserti* and in 10 days in *D. merriami*. The nails hardened by 15 days in both species and the teeth erupted after 5 days in *D. merriami* and in 8 days in *D. deserti*. The teeth were white at first but gradually darkened to a shade of yellow. Eyes opened at about 16 days in *D. deserti* and from 11 to 15 days in *D. merriami*.

Instantaneous growth rates were calculated for various measurements, such as weight, total length, tail length, foot length, and ear length. Both species of kangaroo rats attained average adult weights from 150 to 180 days. Each species reached approximately one-half of its adult weight by 30 days. Early growth was rapid in *D. merriami* but continued more slowly toward maximum weight. *D. deserti* attained maximum adult weight at a slightly earlier age than *D. merriami*. In total length, *D. merriami* grew more rapidly than *D. deserti* throughout the growth period. Both grew to approximately adult lengths by 90 days. The tail of *D. merriami* grew faster than that of *D. deserti* during development. After 90 days *D. merriami* had little tail growth while the tail of *D. deserti* continued to increase slowly in length. The foot showed the fastest development. By 15 days *D. deserti* had 70 per cent of its adult size while *D. merriami* had attained 82 per cent of its adult size. Both species attained maximum size between 50 and

90 days. The ear of *D. merriami* grew slightly faster than that of *D. deserti*. Ears were almost completely grown to adult size in *D. merriami* by 90 days. They continued to grow slowly in *D. deserti*. Size of litter made no appreciable difference in growth rates.

The precocious early development of *D. merriami* may partly explain its larger geographical range.

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Growth, 1961, **25**, 139-149.

ADRENOCORTICOTROPHIN AND MITOTIC ACTIVITY[1]

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Hormones are known to affect the regeneration of vertebrates in a way which usually resembles their effect on growth and morphogenesis. Observations which may seem contradictory or paradoxical are often made, but they are primarily the result of the complicated interdependence of the hormones themselves.

Growth control is a complicated mechanism, and one about which little is yet known. Various theories have however been advanced, one of which was the assumption (33) that an hierarchy of factors exerted an influence on growth, each tissue and organ having a fairly specific factor governing the number and the differentiation of cells. According to the theory, this peripheral system of regulation is affected by another, higher hormonal control, exercised primarily through the pituitary, the thyroid, the gonads, the adrenal cortex and probably also the thymus.

To prove this theory of growth, the authors have devoted their attention to the effect on mitotic division of the different endocrine glands and the hormones secreted by them.

The part played by the adrenal cortex in mitotic activity has received most study, and it has been established that the glucocorticoids inhibit mitoses (3, 8, 13, 31, 33, 35). Cortisone, however, does not inhibit mitoses in all the organs; it may, in fact, have the opposite effect as, for example, on the external orbital gland of the rat if the cortisone is administered with tissue homogenate (36). Deoxycorticosterone seems to have a growth-stimulating effect on the mesodermal tissue (27). The sex hormones secreted by the adrenal cortex, while affecting the development of the sexual structures, usually seem to inhibit the growth of the organism (5, 20).

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Growth has been inhibited, both in vivo and in vitro, by the adrenalin (3). In estrogen-primed rabbits, however, this adrenalin was found to have stimulated very markedly the growth of the mammary tissue (18)—an effect which was annulled by hypophysectomy.

Hypophysectomy has had no apparent definite influence on the mitoses of the epidermis and forestomach of the rat (34); nor does it affect the regeneration of the liver after hepatectomy (1), or the Wolffian lens-regeneration in the newt (25, 29). It does, however, inhibit the regeneration of skull-bones (28).

The pituitary growth hormones encourages anabolic metabolism and the growth of organism (9). Bullough (4), however, maintains that growth hormones have an inhibiting effect on mitoses.

ACTH has been found to stimulate the initial dedifferentiation, or regression of regeneration, while the growth hormone stimulates the progressive phase (19). Schotté and Chamberlain (24) gave ACTH to the newt and found that regeneration was inhibited, a fact which they attributed to increased secretion of glucocorticoids. ACTH also had a retarding influence on regeneration of mucosal lesions of the stomach of the rat (16).

We are unaware of any previous investigations into the actions of adrenocorticotrophin on the mitoses of vertebrates, since the action of ACTH is generally assumed to be identical with the antimitotic action of glucocorticoids. It was observed, however, that the stimulating effect of ACTH on the adrenal cortex not only changed the interrelationship of glucocorticoids but also increased the secretion of mineral corticoids, and affected the pancreatic function, also of importance in tissue regeneration. Attention has, therefore, been concentrated in the present study of the action of ACTH on the mitotic activity of ectodermal and endotermal tissues.

Method

The effect of adrenocorticotrophin on mitotic activity was studied in rat organs and human rectal mucosa.

Rat experiments

Examinations were carried out in three experiments, using 4-month old male Wistar rats. About a week before the experiments the animals were brought to the laboratory to adapt themselves. The

temperature was kept at +20°C, and during the adaption period and the experiment the rats were allowed unlimited food and water.

ACTH injected into intact rats

In *Experiment I*, 10 rats (mean weight 128 g, range 125-140) were given daily intramuscular injections of 4 i.u. of ACTH (Cortrophin-Zinc, Organon) for 12 days. The controls, 10 rats (mean weight 134 g, range 125-140) had no injections.

In *Experiment II*, 10 rats (mean weight 142 g, range 128-156) were given a similar dose of ACTH as in Experiment I, for 10 days. The controls, 10 rats (mean weight 154 g, range 128-162, were given no injections. Specimens of skin and stomach were taken under light ether anaesthesia 24 hours after the final injection, and after the rats in Experiment I had been starved for 24 hours, the rats in Experiment II for 12 hours.

ACTH injected into adrenalectomized rats

In *Experiment III*, an adrenalectomy was performed on 20 rats, and the animals were allowed to drink only 1 per cent saline solution. Two days after the operation, 10 adrenalectomized rats (mean weight 164 g, range 150-180) were given the ACTH treatment described above for 10 adrenalectomized rats (mean weight 167 g, range 146-182) were given daily intramuscular injections of 0.5 ml of saline over the same period. Ten intact rats (mean weight 174 g, range 155-220) received 4 i.u. of ACTH daily for 10 days, and 11 control rats (mean weight 200 g, range 155-220) were given 0.5 ml of saline. Animals which survived the test period were decapitated and, 24 hours after the final injection, specimens were taken from the abdominal skin and gastric mucosa. Rats in agony were decapitated earlier. The specimen stomach removed was opened longitudinally and fastened to cardboard before fixation, as was the specimen of abdominal skin.

Human rectal mucosa

The human material used in the present study consisted of specimens of sigmoid or rectal mucosa from 9 subjects between the ages of 36 and 66 years. Specimens were taken immediately before, and 8 hours after, an ACTH injection (20 i.u. of Cortrophin, Organon) at the same level of the intestine. The ACTH injections

were given intravenously at 8 a.m. and the first mucosal specimen was taken at the time. The next specimen was taken at 4 p.m.

All specimens were fixed with formalin, except for those in Experiment III for which Bouin's fluid was employed. The sections cut at right angles to the skin or the mucosal surface were stained with hemalum-eosin.

The epidermal mitoses were counted in 2,000 cells in successive fields of vision. Starting approx. 1 mm from the limbus, the mitoses of the gastric body mucosa were counted in 4,000 cells over an area of the mucosa 0.12 mm wide (= the diameter of the Zeiss binocular microscope $10 \times 90 \times 1.5$ immersion field of vision in the tissue) at the neck level of the glandular ducts, where their density in rat-stomach is greatest (15).

The mitoses of the sigmoid or rectal mucosa of the human material were counted in 2,000 glandular cells throughout the area of the glandular duct but excluding cells of the superficial epithelium. The t-test was applied in the statistical treatment of the results.

Results

ACTH injected into intact rats

One of the rats given ACTH died in each of the first two experiments. The average weight of the ACTH rats of Experiment I did not change but in Experiment II, weights dropped by an average of 13 per cent.

Mitotic activity in the two experiments increased three-fold compared with the corresponding mitotic rates for the control animals. The increase occurred both in the epidermis and in the gastric mucosa during the test periods of 10 and 12 days. The differences were statistically highly significant ($P<0.001$), as can be seen from Table 1 and Figs. 1 and 2.

Examination of the results also reveals that in Experiment I the mitotic activity in both the rats given ACTH and the controls was definitely lower than in the rats of Experiment II. The only difference experimental conditions under which the rats were kept consisted of a lengthier starvation period prior to decapitation in Experiment I.

A regular feature of the intact rats given ACTH was that the cellular layer of their epidermis was thinner than that of the control

TABLE 1

MEAN OF MITOTIC RATE PER MILLE (± STANDARD ERROR, WITHOUT ACTH AND AFTER ACTH THERAPY) IN THE ABDOMINAL EPIDERMIS AND GASTRIC MUCOSA OF THE RATS

	Epidermis				Gastric mucosa			
Experiment	N:o	Without ACTH	N:o	After ACTH	N:o	Without ACTH	N:o	After ACTH
I	10	2.30 ± 0.49	9	8.72 ± 1.45	10	2.75 ± 0.75	9	10.1 ± 1.26
II	10	4.45 ± 0.61	9	13.50 ± 0.67	10	6.25 ± 1.14	9	15.8 ± 3.20
III Intacts	11	4.00 ±0.93	9	11.61 ± 1.41	11	2.50 ± 0.28	10	9.25 ± 1.33
III Adrenal-ectomized	8	7.25 ± 1.19	9	4.22 ± 0.86	7	6.78 ± 0.69	3	5.16 ± 1.66

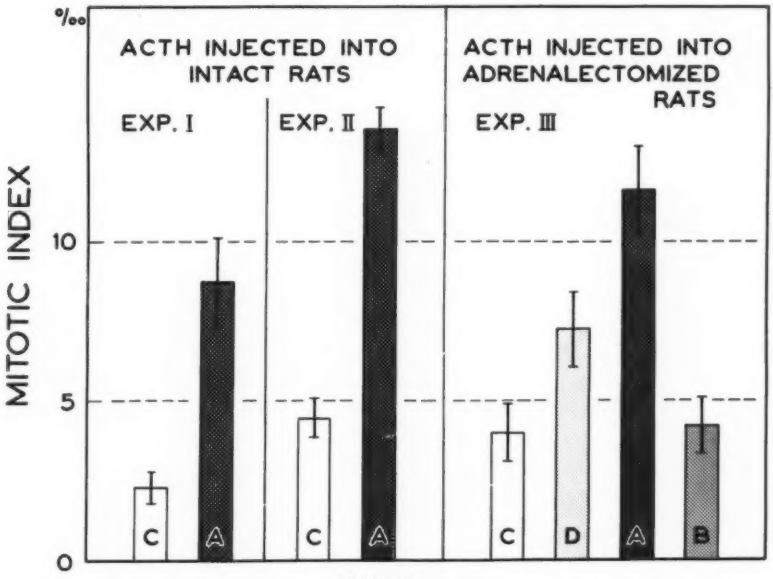

FIGURE 1

Mitotic rate per mille ± standard error in the abdominal epidermis of the rats in three experiments (I:II:III). A: after ACTH application, B: after adrenalectomy + ACTH application, C: intact controls, D: adrenalectomized controls.

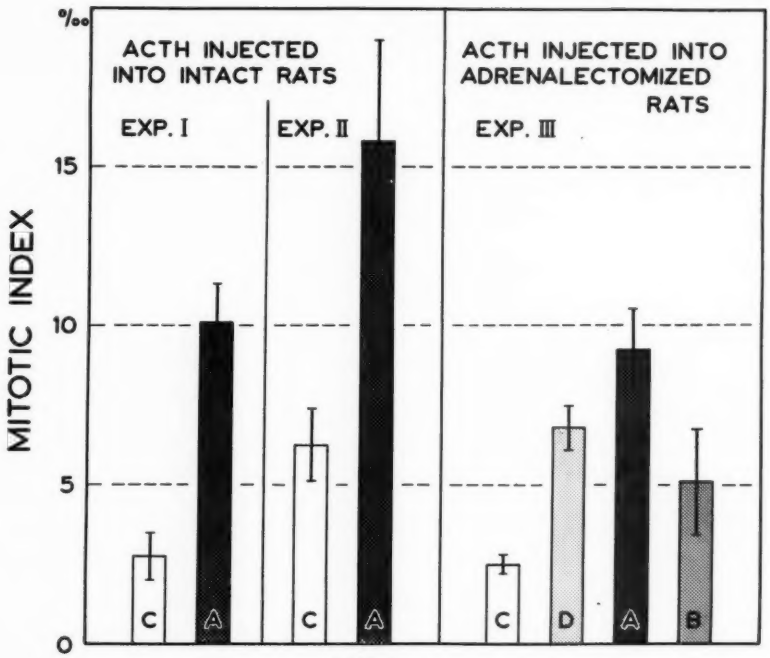

FIGURE 2

Mitotic rate per mille ± standard error in the gastric body mucosa of the rats in three experiments (I:II:III). A: after ACTH application, B: after adrenalectomy + ACTH application, C: intact controls, D: adrenalectomized controls.

animals. The superficial epithelium of the gastric mucosa was irregular, the grouping of the cells in the pit walls was also irregular.

ACTH injected into adrenalectomized rats

Three days before the end of the set period for the third experiment, two of the adrenalectomized rats died and one was killed because of its poor condition. The average loss in weight was 5 per cent. Seven of the adrenalectomized rats treated with ACTH had to be killed, because of their poor condition, 5-1 days before the expiry of the period. The average loss in weight in this group was 9 per cent. The intact rats treated with ACTH were found to be suffering from fatigue at the end of the experiment, and their weights had dropped by 14 per cent.

The mucosal mitoses of the rats killed in agony were not counted since the superficial epithelium of the gastric mucosa of these rats had been damaged. The mucosa of one rat that survived to the end of the experiment was damaged to such an extent that it could not be included in the count. The epidermal specimens of the rats that had to be killed in agony were, however, unblemished.

From Table 1 and Figs. 1 and 2 a study of the result of the mitotic count in Experiment III shows that the variations in the mitotic activity of the epidermis and the gastric mucosa in the groups had a parallel trend, a phenomenon which was also evident in the first and second experiments. Increased mitotic activity was noted after adrenalectomy and the difference between this and the mitotic activity in the gastric mucosa of the control rats was statistically significant ($P<0.01$).

In this experiment intact rats also showed approximately three times the mitotic activity after ACTH therapy ($P<0.001$); such an increase did not, however, occur in rats that were given ACTH after adrenalectomy. The rats of this group which were killed in agony showed a mitotic rate of the epidermis that averaged 4.5 per mille; those that lived to the end of the experiment registered 3.0 per mille.

Human rectal mucosa

In material from 9 patients, the specimens taken from the rectum and, in two cases, those from the sigmoid, showed a mitotic activity of 5.5 ± 1.37 per mille prior to the ACTH injection. In specimens taken after ACTH at the same level of the intestine but not necessarily from the immediate vicinity of the previous specimens, the mitotic activity was 14.9 ± 3.18 per mille. The mitotic activity 8 hours after the ACTH injection was again nearly tripled (Fig. 3), and the difference is statistically significant ($P < 0.01$).

One of the patients had cancer in the aboral part of the intestine; specimens of normal appearance taken outside the tumour showed the highest mitotic activity values of all specimens both before and after ACTH therapy (13.0 and 27.5 per mille).

Discussion

When the results were reviewed, attention was attracted by the fact that ACTH application for 10-12 days to intact rats caused the mitotic rate in the epidermis and gastric mucosa to increase three-

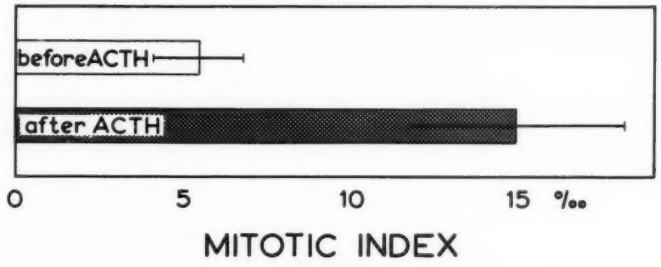

FIGURE 3

Mitotic rate per mille ± standard error in the human aboral intestinal mucosa before, and 8 hours after single ACTH (20 i.u.) injection.

fold. It is probable that this reaction quickly follows ACTH application since in human material also a single injection of ACTH produced a similar rise in the mitotic rate.

No increase in mitoses could be produced by ACTH in adrenalectomized animals.

The rise in mitotic rate revealed in the present study seems to contradict earlier results which show mitotic inhibition by glucocorticoids, which benefited by the adrenal cortex function resulting from ACTH stimulation. Bullough (3) observed that in connection with stress, too, mitotic depression occurred probably as a result of the increased secretion of corticoids.

The results of the study show that adrenocorticotrophin may exert on mitotic activity an influence contrary to that of glucocorticoids. Yet it is also clear that the mitosis-increasing effect of ACTH probably occurs through the adrenal cortex when, as a result of a change in the balance of the steroid hormones secreted by it, mitotic activity may increase in the organism. Another factor contributing towards the change of mitotic activity may be that, in the same way as stress (2), ACTH has an inhibitory effect on thyroid function (14). ACTH has been found to have a synergistic action with the thyroid in normal growth (12, 17, 26) and in the metamorphosis of amphibia (21).

The mitosis-increasing effect of the adrenocorticotropic hormone may not be connected with the growth of the organism, but is perhaps a compensatory phenomenon: cell consumption is perhaps enhanced when metabolism is accelerated under the influence of ACTH. This

is suggested by the weight-loss of the test animals during ACTH therapy, and the narrowing of the epidermal cell layer of the intact rats.

The incomplete differentiation of the superficial epithelial cells of the gastric mucosa may be an indication of increased cell consumption. It is interesting that similar changes in the superficial epithelium of rat-stomach have been achieved by prolonged histamine therapy (6), which stimulates the pituitary adrenal system (23).

Hypophysectomy, which changes the adrenal cortex function in favour of mineral corticoids (10), has a slight mitosis-increasing effect (34). A more marked increase is produced by adrenalectomy, accompanied by an increased genuine secretion of ACTH (23). It is possible that the mitosis-increasing effect on ACTH does not travel via the adrenal cortex alone, but follows other routes as well. Mitoses are inhibited by pituitary growth hormone (4), when the metabolism becomes anabolic.

A decrease in blood sugar is connected with hypophysectomy and adrenalectomy. ACTH, though it raises blood sugar in intact rats, lowers it in adrenalectomized rats (11). Hyperglycemia increases the secretion of insulin, hypoglycemia that of glucagon; both of these have been found to stimulate growth (7, 22). It has been noted that, 24 hours after a meal—irrespective of the kind of food consumed—mitotic activity in the rat's gastric mucosa increases; as the starvation period continues it begins to drop (15). This phenomenon is possibly connected with both the pituitary adrenal system and the pancreatic functions, but more basic study is required to clarify the details.

It is interesting to remember the mitotic stimulating action of ACTH in conjunction with the finding that the mitotic activity of the human epidermis is at its highest between the ages of 40-50 (32), when the functioning of the adrenal cortex is also at its maximum.

It has been generally assumed that ACTH acts on the initial phase of regeneration, i.e. on regression. Needham (19), Schotté & Murphy (25), and Stone & Steinitz (29), however, have found that it also affects progression, a finding which is in favour of growth stimulation.

The zinc content of the prolonged-action ACTH used in the present study hardly played any part in the mitotic activity since a similar mitotic stimulation was achieved in human material with zinc-free ACTH. (The result of human rectal mucosa is relative since mitotic

activity varies at different times of the day and night (37). There were, moreover, no human controls not treated with ACTH.)

Summary

The relationship of ACTH to mitotic activity was investigated in intact rats, adrenalectomized rats, and in human rectal mucosa.

For the rats, the mitotic variations were studied in the mucosa of the glandular stomach, and in the epidermis. With intact animals, a highly significant ($P<0.001$) increase in mitotic activity in the epidermis and the gastric mucosa was observed after ACTH application. Increased mitotic frequency was also observed in adrenalectomized rats. No mitotic stimulation was observed in rats that were given ACTH after adrenalectomy. In the human rectum, too, a definite increase in mitoses was noted after ACTH.

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RETENTION AND TRANSLOCATION OF RADIOACTIVE ZINC BY SALMON FINGERLINGS

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INTRODUCTION

Recent experiments involving the use of radioisotopes have shown that significant amounts of heavy metals taken up by fish from their environment were retained within the fish for extended periods of time. Of the radioactive zinc taken up from treated seawater by the pinfish, *Lagodon rhomboides*, 7 or 8 per cent was retained within the fish over a period of 25 days following removal from the treated seawater (Chipman, Rice and Price, 3). Catfish, *Ictalurus nebulosus*, retained nearly one-half of the zinc-65 taken up from treated fresh water throughout a 7 day observation period after transfer to untreated water (Joyner, 4). Radioactive cerium (Ce^{144}-Pr^{144}), taken up from from water by carp, initially was accumulated by the liver. After one month, about one-half of the liver Ce^{144}-Pr^{144} was lost, a significant amount remaining in the bone (Rudakov, 5).

It was the purpose of this investigation to ascertain the extent to which chinook salmon fingerlings, *Oncorhynchus tshawytscha*, would retain zinc taken up from the water, and to what extent it would accumulate in various parts of the fish.

METHODS AND MATERIALS

Twenty-five actively-feeding chinook salmon fingerlings of common parentage were immersed for twenty-four hours in 5 liters of lake water to which 18.54 microcuries of zinc-65 had been added. The total zinc concentration of the water including the carrier for the radioisotope was 0.2 ppm. During exposure, the water was continously aerated and maintained at a temperature of 14°C. After twenty-four hours the fish were removed from this solution and placed in a 400 liter aquarium supplied with flowing lake water. The

fish were given a surfeit of a liver-fish meal diet twice daily for the balance of the study.

Immediately after their exposure to the radioactive solution, and at 1, 2, 4, 8, 15, 30, and 63 days thereafter, groups of three fish were analyzed for gamma radioactivity. Each whole fish and subsequently the entire vertebral column (less neural and haemal spines) dissected from each fish was separately analyzed. At 8, 15, 30, and 63 days following removal from the radioactive solution, additional determinations were made of the radioactivity in the head and viscera. For radioactivity determinations, a gamma ray scintillation spectrometer with a 3×3 inch, thallium-activated, sodium iodide crystal was used. The maximum counting error for any sample was 8 per cent.

Three control fish, from the same brood stock, were analyzed to determine if zinc-65 was available from the water supply. There was no detectable amount of zinc-65 in these fish.

Results

The radioactivity in the solution in which the fish were immersed was 4.1×10^7 disintegrations per minute. After the fish were removed, 2.4×10^7 d/m remained in solution or suspended in the medium. The rest (about 40%) was either removed by the fish or by sorption to the container and to particles which settled to the bottom. If the average radioactivity per fish on day zero (Table 1) is representative for all 25, then the fish would have taken up approximately 2 per cent of the zinc-65 originally present in the medium.

Observations on the growth of the fish and of the extent and distribution of radioactive zinc within various components of the fish over a 63-day period are summarized in Table 1. Measurements of the radioactivity in the head and viscera were initiated on the eighth day when it became apparent that the salmon were retaining most of the zinc-65. The data show that most of the zinc-65 initially taken up by the fish was retained throughout the entire 63 day observation period. During this time the percent of the radioactivity in the whole fish contributed by the vertebral column increased by a factor of about 2, suggesting translocation of the zinc from other parts of the fish to the vertebrae. The percent of the radioactivity in the whole fish contributed by the head and viscera also increased, but to a lesser

extent than for the vertebrae. If the sums of the average contributions of the vertebral column, head, and visceral mass are subtracted from the radioactivity in the whole fish, the percent of the total body radioactivity contributed by the remainder of the fish (primarily muscle, but including skin, scales, and fins) decreased by a factor of approximately 2 between days 8 and 63.

The degree of retention of radioactive zinc by salmon fingerlings after a 63 day period was not consistent with the results of the experiments with other species of fish previously noted, nor with the 23 day biological half-life for zinc-65 in human beings listed in the National Bureau of Standards Handbook 52 (7, p. 22). The conditions of the present study however were sufficiently different with respect to growth-rate, environmental conditions, and species, that direct comparisons would not be justified.

The mechanisms by which zinc is taken up from water by fish, and subsequently transferred from tissue to tissue within the body of the fish are not fully understood. One theory, presented by Saltman (6) suggests that specific binding sites within various cells accumulate heavy metal ions by a sorption process, independent of metabolic energy. Boyer (1) and Chaberek and Martell (2, p. 450) note that heavy metal ions tend to form stable complexes with serum proteins. The lability of the metal bonds permits exchanges with other substances which have on affinity for metal ions. In vertebrates, the outer surface of the bone appears to serve as an ion-exchange medium capable of taking up large quantities of metal ions whether natural or foreign to the system (Chaberek and Martell 2, p. 451). Metals thus exchanged from serum proteins to the bone may be prevented from undergoing further exchange by the overlayering action of the growing bone.

Summary

Twenty-five chinook salmon fingerlings were immersed for twenty-four hours in 5 liters of lake water containing 0.2 ppm of zinc labelled with 18.54 microcuries of zinc-65. Periodic assays of the gamma radioactivity in the fish showed that the rapidly growing fish had removed approximately 2 per cent of the radioactive zinc from the medium, and had retained nearly all of it for 63 days after transfer to flowing lake water. The increase with time in the percent of the total body radioactivity contributed by the vertebral column, head,

TABLE 1
Zn^{65} IN THE WHOLE BODY, VISCERA, VERTEBRAE AND HEAD OF CHINOOK SALMON FINGERLINGS AT VARIOUS TIMES AFTER EXPOSURE

			Whole Body		Vertebrae		
Fish no.[1]	Water temp. °C	Days after exposure	Wet wt. in g.	Radio-activity in d/m	Dry wt. in g.	Radio-activity in d/m	Per cent[2] of radio-activity in whole body
1			3.21	37,541	.028	651	1.7
2	13.3	0	3.77	53,274	.034	1990	3.7
3			3.52	5,946[3]	.022	1313	22.1
		Avg.	3.50	32,254	.028	1318	4.1
4			3.22	45,179	.020	718	1.6
5	12.8	1	2.67	36,565	.020	896	2.5
6			3.82	57,223	.031	1560	2.7
		Avg.	3.24	46,322	.024	1058	2.3
7			3.02	33,483	.021	1257	3.8
8	13.3	2	3.04	38,496	.019	1257	3.3
9			3.49	35,827	.025	1385	3.9
		Avg.	3.18	35,935	.022	1300	3.6
10			3.35	48,131	.025	1816	3.8
11	13.3	4	2.57	28,405	.019	1087	3.8
12			3.00	42,358	.021	1187	2.8
		Avg.	2.97	39,631	.022	1363	3.4
13			3.81	35,458	.025	1779	5.0
14	15.0	8	2.85	28,015	.023	1324	4.7
15			2.89	24,630	.017	781	3.2
		Avg.	3.18	29,368	.022	1295	4.4
16			4.86	41,035	.033	1801	4.4
17	17.7	15	5.04	41,707	.033	1918	4.6
18			6.24	38,952	.041	1753	4.5
		Avg.	5.38	40,565	.036	1824	4.5
19			9.42	25,432	.074	1562	6.1
20	15.6	30	5.77	36,521	.061	2734	7.5
21			8.52	31,682	.063	2409	7.6
		Avg.	7.90	31,212	.066	2235	7.2
22			14.85	24,422	.148	2955	12.1
23	20.0	63	15.10	27,639	.172	2057	7.4
24			12.44	42,711	.136	2768	6.5
		Avg.	14.13	31,591	.152	2593	8.2
	Growth factor		4.03		5.43		

[1] Fish number 25 jumped out of the aquarium.

[2] The average per cent of the whole body radioactivity contributed by the various parts was obtained by dividing the average d/m for the parts by the average d/m for the whole fish.

[3] This value seems particularly low but is included in all calculations.

TABLE 1 (*Continued*)

Fish no.	Water temp. °C	Days after exposure	Head			Viscera[4]			Remainder
			Dry wt. in g.	Radio-activity in d/m	Per cent of radio-activity in whole fish	Dry wt. in g.	Radio-activity in d/m	Per cent of radio-activity in whole fish	Per cent of total radio-activity
13			.112	9049	25.5	.107	10,286	29.0	
14	15.0	8	.082	7682	27.4	.149	8,615	30.8	
15			.074	6293	25.6	.088	6,011	24.4	41.4
		Avg.	.089	7674	25.9	.115	8,304	28.3	
16			.153	11316	27.5	.138	19,445	47.4	
17	17.7	15	.160	11403	27.3	.166	16,358	39.2	
18			.180	11547	29.6	.186	10,883	27.9	
		Avg.	.164	11422	28.1	.163	15,562	38.4	29.0
19			.255	6944	27.3	.257	8,767	34.5	29.0
20	15.6	30	.163	10525	28.8	.124	12,391	33.9	
21			.233	9591	30.3	.257	13,736	43.4	
		Avg.	.217	9020	28.9	.213	11,631	37.3	27.6
22			.679	7443	30.5	.663	10,434	42.7	
23	20.0	63	.723	7517	27.2	.588	11,388	41.2	
24			.708	14623	34.2	.477	16,456	38.5	
		Avg.	.703	9861	31.2	.576	12,759	40.4	20.2
		Growth factor	7.9			5.0			

[4] Includes heart, kidney, liver, spleen, pancreas and gastro-intestinal tract.

and visceral mass indicates an accumulation of zinc in these parts at the expense of the remainder of the fish.

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EFFECT OF X-IRRADIATION ON DEVELOPING PROTEIN-SYNTHESIZING SYSTEMS[1]

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Recently (1) an attempt was made to ascertain if sub-lethal doses of radiations (400 r to 750 r) would exert any effects on the capacity of embryo chicken liver cells to synthesize certain serum proteins, specifically, a phosphoprotein and a dense lipoglycoprotein, the syntheses of which are stimulated by administration of estrogens. It was found that radiations given 12 through 18 days of incubation were without effect when the chicks were challenged with estrogen on the day of hatching (21 days). Also, when newly hatched chicks were irradiated and estrogenized simultaneously,[2] no decrease in protein synthesis was observed.

However, the question has arisen as to whether in the first case, above, there might not have been recovery from the effects of radiation and in the second case whether the synthesizing mechanisms might not have been largely developed prior to the administration of estrogen and that henceforth it only required triggering by the estrogen to set the bio-synthesis of the unique proteins in motion. In either event, radiovulnerability or the developing synthesizing mechanisms (including the induction and multiplication of ribonucleic acid and enzyme systems) may not have been fairly tested.

Studies were therefore undertaken to determine if the mechanism were present or could be developed in the chick prior to hatching, By

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[2] The rationale behind this was that the ribonucleic acid templates for the protein synthesis would possibly have to be synthesized *i novo* in the nucleus following estrogen stimulation and then pass through the nuclear pores to sites in the reticular endothelium where amino acids could be assembled on the templates. Since the nucleus appears to be more sensitive to radiations than does the cytoplasm (2), the radiation effect might be more damaging just prior to- or during the nuclear synthesis stage.

injection of estrogens into the allantoic sacs of embryos of various stages of development, it was found that the estrogen-induced protein-synthesizing mechanisms were not present and could not be induced prior to the 19th or 20th day of incubation (3).

The above finding permitted the administration of radiations and estrogen at times that were considered to be more critical in terms of the induction period of the estrogen-stimulated protein system than was the case in the original investigation made in 1959 (1).

Materials and Methods[3]

Two modes of attack were employed in these studies. In the first experiment (Experiment 1) embryonated eggs of 18 or 19 days of incubation were injected with 5 milligrams of Estrogenic Substances (Ayerst Co.) each and were then given 600 r (558 rads) of X-irradiation at 18, 18.5, 19, 19.5, 20 or 20.5 days of incubation (Table I). In the second experiment (Experiment 2) the order of treatment was reversed. Embryonated eggs were exposed to 600 r (558 rads) of X-irradiation on the 18th, 19th or 20th day of incubation and were then injected with Estrogenic Substances on the 19th, 20th or 21st day (Table I). Appropriate control groups received: estrogen but no X-irradiation; X-irradiation but no estrogen; neither estrogen nor X-irradiation. The X-irradiation factors were 250 Kvp, 15 ma, 35 cm TOD, 0.21 mm Cu inherent, plus 0.5 mm parabolic Cu, plus 1.0 mm Al filters, HVL = 1.9 mm Cu, centers of parabolic filter. The dose rate was 78 r per minute as measured in air. Both chicks and eggs were well centered in the field, the eggs standing on the small ends in egg boxes and the chicks standing in boxes covered by celluloid.

[3] Serum proteins elicited by estrogen treatment include serum X_1-phosphoprotein (Common and Mok's phosvitin is a derivative of X_1(4)), serum X_2-lipoglycoprotein (this is probably very similar to the lipovitellin of serum) and a lighter lipoprotein. In the chick these are characterized by Svedberg constants S8.5, S16.5 and S_f15-100, respectively. The phosphoprotein has been shown to carry most of the elevated calcium in the sera of these fowl. The lipoglycoprotein contains approximately 20 per cent lipid, mainly phospholipid, and the lipid moiety of the lighter lipoprotein (80% of the total molecule) consists mainly of sterol ester and phospholipid with varying amounts of triglyceride. Except in the case of the laying hen, these proteins are rarely present in the serum in significant concentration. Previous studies have shown that administration of estrogen results in an increase of the lighter lipoproteins (S_f15-100) at relatively early stages of embryonic development.

Since the effects of X-irradiation in decreasing serum concentrations of albumin and normal dense lipoproteins are well documented in previous publications (1, 5), since the production of estrogen-induced light lipoproteins would not be expected to be greatly effected by radiations and since serum X_2-lipoglycoprotein is less reliably measured in low concentrations (such as obtain in estrogenized chicks) than serum phosphoprotein, only serum X_1-phosphoprotein measurements are presented in this report.

The amount of serum calcium in excess of the normal value (11^{-13}mg %) was taken as the most reliable reflection of the amount of X_1-phosphoprotein present in the serum. It has been shown that in estrogenized birds the serum calcium above this value is largely (>90 per cent) bound to the special phosphoprotein (6).

Results

As can be determined from inspection of Table I, no depressing effect on the synthesis of serum X_1-phosphoprotein could be detected despite concerted efforts to select optimum timing and sequence with respect to irradiation and estrogen treatment. To the contrary, several determinations of X_1-phosphoprotein levels in groups of irradiated chicks revealed concentrations of this material that were significantly above the mean of the chicks receiving estrogen only. (Hematocrit values were approximately equal in both irradiated and non-irradiated groups.) These elevated phosphoprotein levels in the irradiated groups, coupled with slightly depressed calcium readings in embryos receiving only X-irradiation, contributed to a 28 per cent over-all increase above the mean in the groups receiving estrogen only but the probability of a significant difference between the two groups was only approximately 0.1. The individually elevated values did not occur as a consequence of any specific sequence of treatment.[4]

Discussion

In discussing the reasons for the lack of effect of x-irradiation upon induction of the x_1-phosphoprotein synthesizing mechanism, it may be useful to compare this radiation-refractory system with the apparently *radiosensitive* alpha lipoprotein and albumin synthesizing mechanisms.

[4] So far as could be detected, no effect of irradiation was exerted upon the production of serum-X_2-lipoprotein or upon the lighter lipoproteins induced by estrogen treatment.

TABLE I
TOTAL SERUM CALCIUMS

Exp. number	Age est.* given	Age X-rays given	Age bled	Food	Hematocrit (% red cells)		Total serum calcium (mg %)			
Control	None	None	23**	Mash, ad lib.		14.7				
Control	None	None	24	Mash, limited		11.2				
Control	None	None	26	Mash, limited		11.2				
Control	None	None	26	Mash, limited		11.2				
Control	None	None	24	Mash, limited		15.2				
Control	None	None	24	No food		14.0				
Control	None	18	24	No food			9.8			
Control	None	19	24	Mash, limited			14.0			
Control	None	20	24	No food			8.6			
1	18	None	23	Mash, limited				16.0		
1	18	None	23	Mash, limited				15.4		
1	18	18	23	Mash, limited						23.4
1	18	19	23	Mash, limited						29.6
1	19	None	23	Mash, ad. lib.				20.6		
1	19	19	23	Mash, ad. lib.						15.8
1	19	20	23	Mash, ad. lib.						16.8
1	19	20	23	Mash, ad. lib.						17.1
1	19	19	23	Mash, limited						20.1
1	19	20	23	Mash, limited	48					34.0
1	19	None	23	Mash, limited		52		20.3		
1	19	20	23	Mash, limited	48					22.3
1	19	20	23	Mash, limited	52					18.7
1	19	None	23	Mash, limited		47		20.8		
1	19	None	23	Mash, limited				25.9		
1	19	19(700r)	23	Mash, limited						23.6
1	19	19(700r)	23	Mash, limited						21.0

TABLE I (*Continued*)

Exp. number	Age est.* given	Age X-rays given	Age bled	Food	Hematocrit (% red cells)			Total serum calcium (mg %)		
1	19	None	23	Mash, limited					17.9	
1	19	18½	23	Mash, limited						23.7
1	19	19½	23	Mash, limited						20.8
1	19	20½	23	Mash, limited						22.4
2	19	None	23	Mash, limited					25.6	
2	19	18	23	Mash, limited						30.5
2	20	None	23	Mash, limited					22.4	
2	20	19	23	Mash, limited						29.2
2	21***	None	23	Mash, limited					27.7	
2	21	19	23	Mash, limited						28.5
2	21	None	23	Mash, limited		53			26.8	
2	21	20	23	Mash, limited	45					25.8
2	21	None	23	Mash, limited		45			29.9	
2	21	20	23	Mash, limited	42					41.5
					Mean = 47	Mean = 49	Mean = 12.9	Mean = 10.8	Mean = 22.4 − 12.9 = 9.5	Mean = 24.1 − 10.8 = 13.3

* Estrogen.
** 2 days post-hatching.
*** Day of Hatching.

Although *alpha lipoproteins* are normally increased twofold in the chicken embryo's plasma just at the time of hatching, 465 rads of ionizing radiation administered to the whole body of the chicken embryo three days prior to hatching causes a failure of this increase to take place (7). At earlier stages of development, radiation-induced inhibitions of increase are barely detectable; and, if radiations are administered following hatching, there occur increases—rather than decreases—in the serum levels of alpha lipoproteins.

These differing results may, perhaps, be explained by the (probably indirect) effects of radiations on the lipid metabolisms of the liver cells. Gould (8) has shown that cholesterol synthesis is increased many-fold in the liver of irradiated rats. It is therefore not surprising that in cases of radiation-induced decreases in the normal amounts of alpha lipoproteins, there occur concomittant increases of the abnormal beta lipoprotein fractions (S_f10-100) which contain considerably more cholesterol ester. On the other hand, when the alpha lipoproteins are *increased* in the plasma (as they are if the chick is irradiated following hatching), these increases may again be due to increases in cholesterol synthesis; but by this time the basic serum lipoprotein synthetic pattern has shifted so far in the direction of large amounts of alpha (dense) lipoproteins that this fraction apparently has to carry the brunt of the abnormal lipid. In favor of such a hypothesis is the fact that the increase in dense lipoprotein following irradiation is observed to be greater in the lighter -S7 component than in the -S4.5 component. In fact, all of the above observations point to a greater derangement of the lipid economy of the cell following radiation exposure than of its polypeptide synthesizing mechanisms.

The depressing effect of ionizing radiations on production of *serum albumin* may possibly be explained by the role of serum albumin as a "buffer" system in the liver cell's scheme of serum protein synthesis. In a sense the synthesis of albumin by the liver is analogous to the production schedule of a boat factory where row boats constitute the staple product, keeping the men and machinery at work; but whose production may be on a moment's notice curtailed by the placing of an order for an expensive yacht. Although serum albumin undoubtedly plays some important roles in the organism's physiology (such as the transport of unsaturated fatty acids, cholesterol, steroids and other vital substrates) it can get along without any albumin (such as it does

as an early embryo and in various of the more primitive vertebrates) or at least, with very little. In cases of starvation and following injection of estrogen (in birds) the serum albumin is considerably depressed while other serum proteins remain at relatively high levels. These observations lead to the notion that serum albumin is ordinarily synthesized in excess of the organism's immediate requirements and that when an emergency arises that calls for increased production of some other component within the cell (not necessarily a serum protein) its synthesis is automatically interrupted. Since near-lethal doses of ionizing radiations provoke various primary disturbances of liver metabolism resulting in increases of synthesis of certain lipids, etc., it would be expected that these increases be reflected in part by a depression of albumin synthesis.

In contrast to the organism's "lackadasical" approach to the production of serum albumin stands the concerted effort of the estrogenized (or egg-laying) fowl to synthesize as much of the estrogen-induced serum proteins as fast as possible. When reproduction is the goal, the metabolism of the bird is altered in a most immoderate fashion. The chicken liver cells funnel their resources so strongly into the production of proteins that will serve as nutrients for the prospective embryo that other syntheses (such as those of not only albumin but alpha lipoproteins and mucoproteins as well) suffer by contrast. If this concentration of purpose may be extrapolated back to those systems that are most likely to be injured by radiations, it appears quite possible that so long as the cell is not killed by irradiation (and the liver cells are not destroyed by the dose-range under consideration) it will continue to elaborate the estrogen-stimulated proteins at normal rates, even at the expense of synthesis of other proteins.

It appears unlikely that, within the dose range employed, any effect of radiation on serum protein synthesis is exerted through the genes ultimately responsible for this synthesis. For this to take place specific mutations would have to occur in a relatively high proportion of the irradiated liver cells. This simply does not occur. During, especially, the induction period of an inducible synthetic system, such as the one under discussion, it could be expected that enzymes involved in this process would be partially altered in concentration or inactivated (directly or indirectly) by ionizing radiations or associated oxidizing radicals. However, in such a case, partial elimination of an enzyme

system might not be sufficient to alter the observed rate of production of a protein, particularly when the rate limiting factor of the synthesis might be some component other than the radio-sensitive enzymes.

Conclusion

No depressing effect of 558 rads of X-irradiation on the developing synthetic mechanisms for estrogen-induced serum X_1-phosphoprotein could be detected, despite concerted efforts to select optimum timing and sequence with respect to irradiation and estrogen treatment. Possible reasons for the lack of sensitivity of this protein synthesis system, as compared to the serum albumin and dense (alpha) lipoprotein mechanisms, are discussed.

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CARBOHYDRATE METABOLISM IN THE LENS OF THE AGING RAT*

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Experiments recently concluded in this laboratory have demonstrated an active Hexose Monophosphate Pathway in the young and rapidly growing ocular lens derived from rats aged 26-32 days, Lerman (6 and 7) and Lerman and Ishida (9). When such animals are put on a cataractogenic galactose diet, Yudkin and Arnold (13), lens vacuoles become apparent after 8-9 days and dense opacification of the lens occurs after 12-15 days on this diet. Although the mechanism whereby galactose exerts its cataractogenic action has not been fully defined, the aforementioned experiments have indicated that galactose-1-phosphate acts as, or gives rise to, a specific inhibitor of glucose metabolism in the rat lens. A specific inhibition of the enzyme glucose-6-phosphate (G-6-P) dehydrogenase develops after such animals are maintained on the high galactose diet for 2-3 days, Lerman (4). This inhibition is paralleled by a marked decrease in the activity of the Hexose Monophosphate Pathway as reflected by the recovery of $C^{14}O_2$ from lenses incubated with glucose-1-C^{14} (G-1-C^{14}), Lerman (5). A decline in ATP also occurs at this time and by day 9-10 (when lens vacuoles become evident) there is little or no ATP present in these lenses, Lerman and Ishida (9), and the synthesis of soluble lens protein ceases after the rats have been on the galactose diet for 6-8 days, Lerman (6) and Dische (2).

As the rat ages the length of time required to induce a cataract by means of such feeding experiments increases progressively. When rats 85-100 days of age are placed on the high galactose diet opacification of the lens does not develop until these animals have been

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maintained on such a regime for 9-10 weeks. Thus the age of the animals is evidently an important factor in the ease and rapidity with which they develop galactose cataracts. Since galactose may rapidly inhibit the Hexose Monophosphate Pathway of glucose metabolism and apparently does not interfere with anaerobic glycolysis (as measured by lactate production, Wachtl and Kinsey (11) and Pirie and Van Heyningen (10)), it seemed likely that the lens of the mature rat may differ from that of the very young animal with respect to its relative rate of utilization of the direct oxidative pathway or anaerobic glycolysis as its major pathway of glucose metabolism.

In order to investigate this hypothesis the relative rates of activity of these 2 routes of glucose metabolism were studied in lenses derived from rats aged 28-32 days and from rats 85-100 days of age. The activity of G-6-P dehydrogenase was also determined in lenses from 2 similar groups of animals and this enzyme assay was repeated at specific intervals on lenses derived from one group of young rats and a second group of older rats, both of which had been maintained on the cataractogenic galactose diet for periods of time ranging from 3-70 days.

Materials and Methods

Holtzman strain white male rats, 28-32 days old, were used in the experiments involving the rapidly developing lenses from young animals. The same strain of white male rats, aged 85-100 days, was employed as the source of the mature lenses. Henceforth, lenses derived from rats aged 28-32 days will be referred to as young lenses, while those derived from rats aged 85-100 days will be referred to as the mature lenses.

A. *Experiments with labelled glucose*

Glucose-C^{14} labelled in the number one or number six carbon atom (Nuclear Chicago) was employed as the substrate; the specific activity of G-1-C^{14} was 2,600 cpm/micromole, while G-6-C^{14} had a specific activity of 2,700 cpm/micromole. A single mature lens from each of 20 rats was employed for this experiment. Half of these lenses were individually incubated with G-1-C^{14} and the remaining 10 lenses were incubated with G-6-C^{14}. The apparatus and the methods employed have been described in a previous communication, Lerman (5). The young lenses were treated in a similar manner. Since the recovery of $C^{14}O_2$ from both the young and mature lenses incu-

bated with G-6-C^{14} was quite low, the counts were taken for 30 minute intervals per planchet. A sufficient amount of data have been accumulated in these experiments and in experiments previously reported to indicate that these values, while low, are meaningful. Furthermore, six experiments were done in which 2-4 lenses were pooled, the recovery of $C^{14}O_2$ from G-6-C^{14} was increased by a similar factor.

B. *G-6-P dehydrogenase activity in the lenses from young and mature rats maintained on the control and high galactose diet*

Of the 20 lenses remaining from each group of animals employed in the previous experiment, 11 mature lenses and 10 young lenses were used in the G-6-P dehydrogenase assay. This enzyme was assayed according to the method of Glock and McLean (3). Since the soluble protein content of the mature lenses is approximately 40-60% higher than that of the young lenses, the enzyme activity could not be based on such a variable parameter. The remaining lenses (9 mature and 10 young) were therefore assayed for their individual RNA concentrations and the activity of G-6-P dehydrogenase will be expressed on the basis of the mean value of RNA per lens. The content of RNA per lens remained remarkably constant in the lenses of both the control and galactose-fed rats during the experimental period. RNA was determined by the orcinol reaction, Brown (1). All the lenses employed in these experiments and in the studies described in section A were fresh organs.

Ten Holtzman strain white male rats aged 85-100 days were employed in the experiment to determine the effect of galactose feeding on G-6-P dehydrogenase activity. They were all placed on the galactose diet and 2 animals were killed at specific intervals as shown in Figure 1. Their lenses were immediately removed, weighed and homogenized in individual Dounce microhomogenizers (containing 1 ml of distilled water), kept in an ice water bath. The insoluble protein fraction was removed by centrifugation in a refrigerated centrifuge. An aliquot (0.2 ml) from each supernatant solution was employed for the G-6-P dehydrogenase assay and the remainder was used for RNA determinations.

Results

A. The results of the experiments in which individual lenses were incubated with G-1-C^{14} or G-6-C^{14} are shown in Tables I and II. In the young lenses, the mean C_1/C_6 ratio of 14.4 indicates that this organ utilizes the Hexose Monophosphate Pathway as the preferential pathway for the combustion of glucose to CO_2. In the mature lens there is a marked fall in the amount of $C^{14}O_2$ recovered from G-1-C^{14}, while the corresponding value of $C^{14}O_2$ recovered from G-6-C^{14} is somewhat higher than the values obtained from the young lens.

TABLE I
EXPERIMENT WITH LENSES DERIVED FROM RATS AGED 28-32 DAYS

Lens number	$C^{14}O_2$ recovered from G-1-C^{14} in cpm/gram wet weight	$C^{14}O_2$ recovered from G-6-C^{14} in cpm/gram wet weight	C_1/C_6
1 & 2	1600	140	11.4
3 & 4	1060	40	26.5
5 & 6	780	30	26.0
7 & 8	710	80	8.9
9 & 10	1580	150	10.5
11 & 12	1140	100	11.4
13 & 14	960	140	6.9
15 & 16	1500	110	13.6
17 & 18	1430	100	14.3
19 & 20	1460	lost	
Mean	1222	99	14.4
S.D.	± 334	± 43	± 7.08

TABLE II
EXPERIMENT WITH LENSES DERIVED FROM RATS AGED 85-100 DAYS

Lens number	$C^{14}O_2$ derived from G-1-C^{14} in cpm/gram wet weight	$C^{14}O_2$ derived from G-6-C^{14} in cpm/gram wet weight	C_1/C_6
1 & 2	320	290	1.1
3 & 4	363	370	1.0
5 & 6	516	168	3.1
7 & 8	456	162	2.8
9 & 10	432	lost	
11 & 12	534	180	2.9
13 & 14	230	105	2.2
15 & 16	270	100	2.7
17 & 18	312	132	2.4
19 & 20	306	150	2.0
Mean	374	184	2.02
S.D.	± 104.6	± 81.8	± 0.783

The C_1C_6 ratio of approximately 2 in the mature lens indicates that the activity of the direct oxidative pathway may have diminished considerably.

B. The results of the G-6-P dehydrogenase assay in the young and mature lenses are shown in Table III. The mean activity of this enzyme was found to be 2.26 units in the young lens compared with 1.99 units in the mature lens. A statistical analysis of these data revealed that there was no significant difference in the activity of G-6-P dehydrogenase between these two groups of lenses ($P > 0.05$).

TABLE III

Lens number	G-6-P dehydrogenase activity in the mature lens	G-6-P dehydrogenase activity* in the young lens
1	2.90	2.67
2	2.93	2.02
3	1.95	2.55
4	1.66	1.64
5	2.00	2.53
6	2.40	1.34
7	1.76	1.86
8	2.36	2.02
9	2.36	1.52
10	2.24	1.70
Mean	2.26	1.99
S.D.	± 0.43	± 0.46
	$P > 0.05$	

* G-6-P dehydrogenase activity is expressed as micrograms TPN reduced per hour per microgram RNA per lens.

The results of the G-6-P dehydrogenase assay in the mature lenses derived from rats maintained on a cataractogenic galactose diet are shown in Figure 1. The inhibition of G-6-P dehydrogenase becomes apparent after the animals have been maintained on the galactose diet for 3-5 days and the decline in the activity of this enzyme closely parallels that observed in the experiments with the young lenses as reported in a previous communication, Lerman (6). Although vacuoles became apparent in the young lenses after 9 days on the experimental diet and dense opacification occurred by day 12-15, the length of time required for vacuolization and cataract formation to occur in the mature lens was 65-70 days.

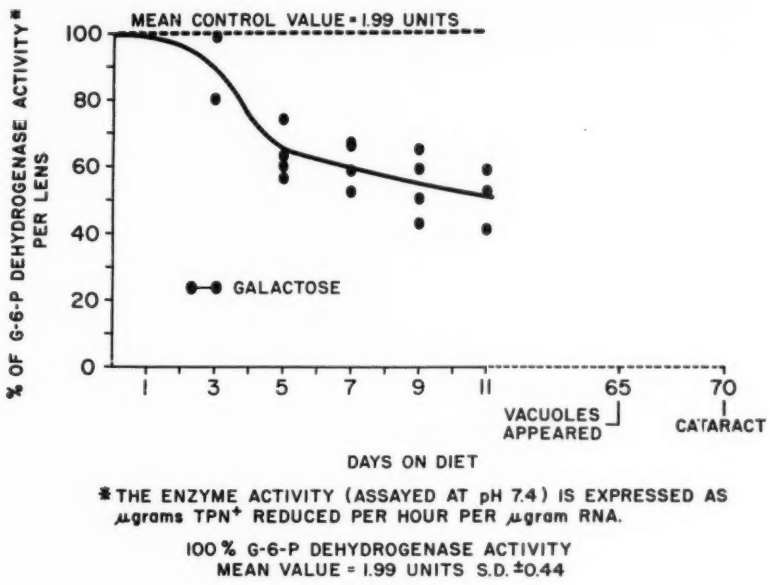

FIGURE 1

The effect of a high galactose diet on the activity of G-6-P dehydrogenase in the ocular lens of the rat 85-100 days of age.

Discussion

The experiments based on the C_1/C_6 ratio of $C^{14}O_2$ recovered from the young lens incubated with glucose-1-C^{14} and glucose-6-C^{14} indicate the possibility of an active direct oxidative pathway in this organ. However, in view of the questions raised by Wood (12) regarding the validity of such an experimental technique as a basis for determining the presence and degree of activity of the Hexose Monophosphate and the Embden-Meyerhof Pathways of glucose metabolism, an attempt was made in these studies to circumvent one of the possible sources of error. This was done by incubating these lenses for 21 hours compared with the usual 2-4 hours employed by other investigators. Furthermore, the resultant C_1/C_6 data although indicative of the presence of an active Hexose Monophosphate Pathway in the rapidly developing rat lens should not be considered as con-

clusive evidence. Thus other experiments were performed which indicated a high level of activity of G-6-P dehydrogenase in this organ, and the TPNH/TPN ratio in such lenses was 4.5 to 1 compared to a DPNH/DPN ratio of approximately unity, Lerman (8). In view of a high TPNH/TPN and C_1/C_6 ratio it is probably not unreasonable to assume that the young rat lens possesses an active Hexose Monophosphate Pathway. In the mature lens, both the C_1/C_6 and TPNH/TPN ratios fall to a value close to unity, thus indicating that this direct oxidative pathway is no longer as active in this organ.

Although the activity of the Hexose Monophosphate Pathway apparently decreases as the lens ages, the activity of the enzyme G-6-P dehydrogenase (which catalyzes the first step of glucose oxidation via this route) apparently remains unchanged. Furthermore, the ease and rapidity with which this enzyme can be inhibited (in vivo) by a high galactose diet is also independent of the age of the animal. In both the young and mature lens, there is a significant decline in the activity of G-6-P dehydrogenase after the animals have been maintained on the cataractogenic galactose diet for 3 days, and there is a similar and progressive decline with time. But cataract formation does not occur until the older rat (age 85-100 days) has been maintained on the diet for 65-70 days in contrast to the relatively short period of time required (12-15 days) for dense opacification to develop in the young rat (28-32 days of age).

These experiments indicate that the relative importance of one pathway of carbohydrate metabolism in the lens of the rat may be closely correlated with the respective age and degree of development of the organ. The Hexose Monophosphate Pathway is apparently an active pathway in the metabolic economy of the young and rapidly developing lens of the rat and its susceptibility to galactose as a cataractogenic agent may depend to some extent on this factor. As the lens matures, the activity of the Hexose Monophosphate Pathway diminishes considerably. Since galactose-1-phosphate acts as or gives rise to an inhibitor of the enzyme G-6-P dehydrogenase in both the young and mature rat lens, the consequences of a marked inhibition of the pathway may not be as severe in the mature lens as compared with the young lens.

Summary

The ocular lens from the mature rat (age 85-100 days) differs from that of the young 28-32 day old rat in the activity of the Hexose Monophosphate Pathway of glucose metabolism. This pathway is responsible for most of the combustion of glucose to CO_2 in the young lens but decreases markedly in activity as the animal ages.

The cataractogenic action of galactose is a very rapid one in the young lens while it requires 6-7 times as long for a cataract to appear in the 85-100 day old rat maintained on a high galactose diet. The enzyme glucose-6-phosphate dehydrogenase shows a parallel rapid and marked inhibition in the lenses from both the young and older rat maintained on the cataractogenic galactose diet.

The metabolism of glucose by the ocular lens via the direct oxidative pathway differs markedly in these 2 groups of rats. The lens of the young rat may be more dependent on the Hexose Monophosphate Pathway and an inhibition of this pathway may be an important factor in the pathogenesis of the experimental galactose cataract. In the lens from the older rat a similar inhibition of the enzyme catalyzing the first step of glucose oxidation via the direct oxidative pathway is apparently of less consequence.

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PROTEIN ANABOLISM AS A FACTOR AMELIORATING THE EFFECTS OF EARLY THYROID DEFICIENCY

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INTRODUCTION

Much of our present understanding of the inter-relationship between the growth-promoting effects of thyroid and pituitary secretions has been inferred from a series of experimental observations based on the effects of neonatal hypophysectomy (Walker, Simpson, Asling and Evans 1950; Asling, Walker, Simpson, Li and Evans, 1952; Walker, Asling, Simpson, Li and Evans, 1952) and thyroidectomy (Salmon, 1936; Scow and Simpson, 1945; Ziskin, Salmon and Applebaum, 1940;) and of subsequent medication with thyroxine and somatotrophin given either alone or in combination (Evans, Simpson and Pencharz, 1939; Salmon, 1938; 1941; Scow and Marx, 1945; Scow, Simpson, Asling and Evans, 1950). In summary it may be said that, judged in terms of total body weight and the weight of non-endocrine viscera, the administration of anterior pituitary growth hormone will in large measure restore the growth of neonatally thyroidectomised rats, the effect being greatly enhanced by the presence of small thyroid remnants or by concomitant treatment with thyroxin. On the other hand, these procedures fail to induce the changes in bodily form and skeletal structure which characterise the transition from infant to adult. The action of somatotrophin has thus come to be regarded as one concerned with incremental growth mediated through the body's capacity to synthesise protein; thyroid hormone on the other hand, while possibly playing the role of synergist to somatotrophin would seem to be more specifically involved in maturational phenomena.

In two important respects, however, the information provided by this group of investigations is lacking. The first concerns the pos-

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sible influence of the age at which the experimental procedures are carried out; for in nearly all studies involving the administration of protein anabolists to hypo- or athyroid individuals either the primary thyroidectomy or the subsequent experimental treatment has been undertaken after weaning by which time, in the rat at least, much of the maturational influence of thyroid hormone may be regarded as having waned. Second, only passing attention has been paid in these studies to the development of the central nervous system, most being concerned only with the weight of the brain. Conclusions based on such limited criteria can be misleading, however, and while the growth of the brain is admittedly less affected either by neonatal hypophysectomy (Asling et al., 1952) or thyroidectomy (Scow and Simpson, 1945) than is that of the body and responds correspondingly little to subsequent medication (Scow et al., 1949) more recent histological examination has shown that, structurally, the brain of the cretinoid individual differs in many respects from that of the normal. For instance, as a result of giving the goitrogen methyl thiouracil to newborn rats, the normal proportionality of the length and width of the brain is altered (Eayrs and Taylor, 1951), the pattern of vascularity in the cerebral cortex is modified (Eayrs, 1954), the cortical perikarya are smaller and their packing density increased (Eayrs and Taylor, 1951) and both the axonal and dendritic components of the neuropil are hypoplastic (Eayrs, 1955) a factor which results in a marked reduction in the probability of axo-dendritic interaction between neurones (Eayrs, 1959). Some, at least, of these effects are similar to those seen after inanition (Eayrs and Horn, 1955) when the synthesis of protein is prevented by dietary deficiency, and an enhanced cerebral growth has been recorded as a result of giving growth hormone during pregnancy (Zamenhof, 1942). It seemed possible, therefore, that protein anabolic agents might have a more significant influence than could be revealed in terms of weight alone.

The experiments which form the subject of this paper have been designed to answer two questions: first, the extent to which the neurohistological changes which result from chemical thyroidectomy are attributable to thyroid deficiency as opposed to some side effect on the part of the goitrogen used; and second, whether the structural changes which take place in the brain between birth and maturity (Eayrs and Goodhead, 1950) are to be regarded as a phenomena

of incremental growth or of maturation. If the former, then it might be expected that the developmental changes seen in the brain of the neo-natally thyroidectomised rat could be prevented by giving substances known to have nitrogen-retaining properties; if the latter, then only thyroid hormone should be capable of rectifying the deficiency. To this end the effect of neo-natal radio-thyroidectomy on cerebral development, and the capacity of purified somatotrophin to ameliorate these effects, has been examined. In addition since the steroid anabolist methylandrostenediol (MAD) has been shown to restore the rate of growth in thyroidectomised rats (Salgado and Selye, 1954) the effect of giving both this substance and the related 19-non-testosterone (19 NT), also known to have nitrogen-retaining properties (McSwiney and Prunty, 1957), has been studied.

Materials and Methods

The animals used for these experiments were taken from litters of the Birmingham strain of albino rats. On the day of birth the sex of the young was determined in each litter and animals whose sex was present in the lesser number were discarded. Where equal numbers of each sex were born the males were preferred. The animals retained were divided into two groups of unequal size: two rats were selected at random to serve as controls; the others were radio-thyroidectomised by the method of Goldberg and Chaikoff (1949). On the following day, and daily thereafter, half the rats in each group were subcutaneously injected as under, the remaining half receiving the same volume of the vehicle:

Somatotrophin (a highly purified preparation distributed by the National Institute of Health)	300 µg (24 litters)
Methylandrostenediol	750 µg (7 litters)
19-nor-testosterone	350 µg (6 litters)

The growth hormone was made up as a 0.3% solution in normal saline; the steroids were first dissolved in 50% propylene glycol to form a 10% solution which was subsequently diluted to a strength suitable for giving the required dosage in approximately 0.1 ml of solution.

Between the 14th and 24th days the rats in the four groups were tested in the manner described by Eayrs and Lishman (1955) for the elicitation of the placing reaction. All animals were weighed on the 24th day, anaesthetised with tribromoethanol and killed by decapitation after the trachea and larynx had been removed for subsequent histological examination for thyroid residues. Their brains were then removed and fixed in preparation for processing either by the method of Nonidez (1939) for showing axons, or by the Golgi-Cox technique (Sholl, 1953) for dendrites. At a later stage the brains were weighed and their length and width measured along the axes previously used by Eayrs and Taylor (1951). Quantitative histological data relating to the densities of axons and dendrites within the neuropil were obtained, as described by Eayrs (1955), from sections cut through that part of the sensori-motor cortex essential for the mediation of the placing reaction (Brooks, 1933). All measurements were treated by the appropriate method of variance analysis with the exception of those for the placing reaction. The mean age at which this response emerged was determined by the methods of probits (Finney, 1952) but owing to the unreliability of fiducial limits obtained from correlated data the significance of differences associated with the several treatments was estimated by the non-parametric method described by White (1952).

Data derived from experimental rats in which remnants of thyroid tissue were found were rejected from the analyses.

Results

1. *Effects of radiothyroidectomy.*

The effects of neonatal thyroidectomy induced by giving radio-iodine were essentially similar to those described following surgical ablation (Salmon, 1938; Scow and Simpson, 1945) or the chronic administration of methyl thiouracil (Eayrs and Taylor, 1951; Eayrs 1955) and are therefore summarized but briefly. Thus the growth of both body and brain was retarded, that of the body more severely than that of the brain, while changes in the shape of the brain were such as to give rise to a structure abnormally wide in relation to its length (Fig. 1). Cerebral cortical structure was at the same time modified. The axonal network proved hypoplastic in all laminae (Fig. 2) but the effect was disproportionately more pronounced in

PERCENTAGE INCREASE OR DECREASE

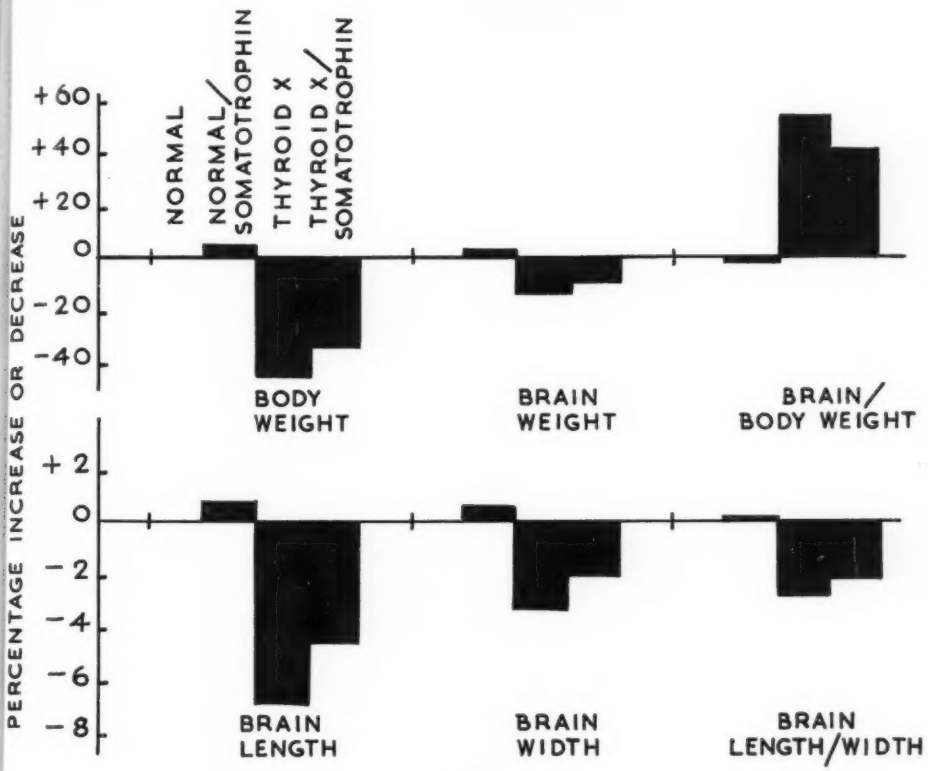

FIGURE 1

Changes in the growth of body and brain of normal and neonatally thyroidectomised rats given purified somatotrophin. For ease of comparison the data are expressed as a percentage increase or decrease related to the normal individual taken as zero.

the internal granular layer (lamina 4). The development of dendrites was also impaired. Although the number of primary dendrites arising from the perikaryon was little affected the amount of branching was reduced at all distances from the cell body (Fig. 3A) while the mean length of dendrites was significantly increased (Fig. 3B). These changes resulted in an overall decrease in dendritic density and a shift to the left of the curve representing the pattern of decay of

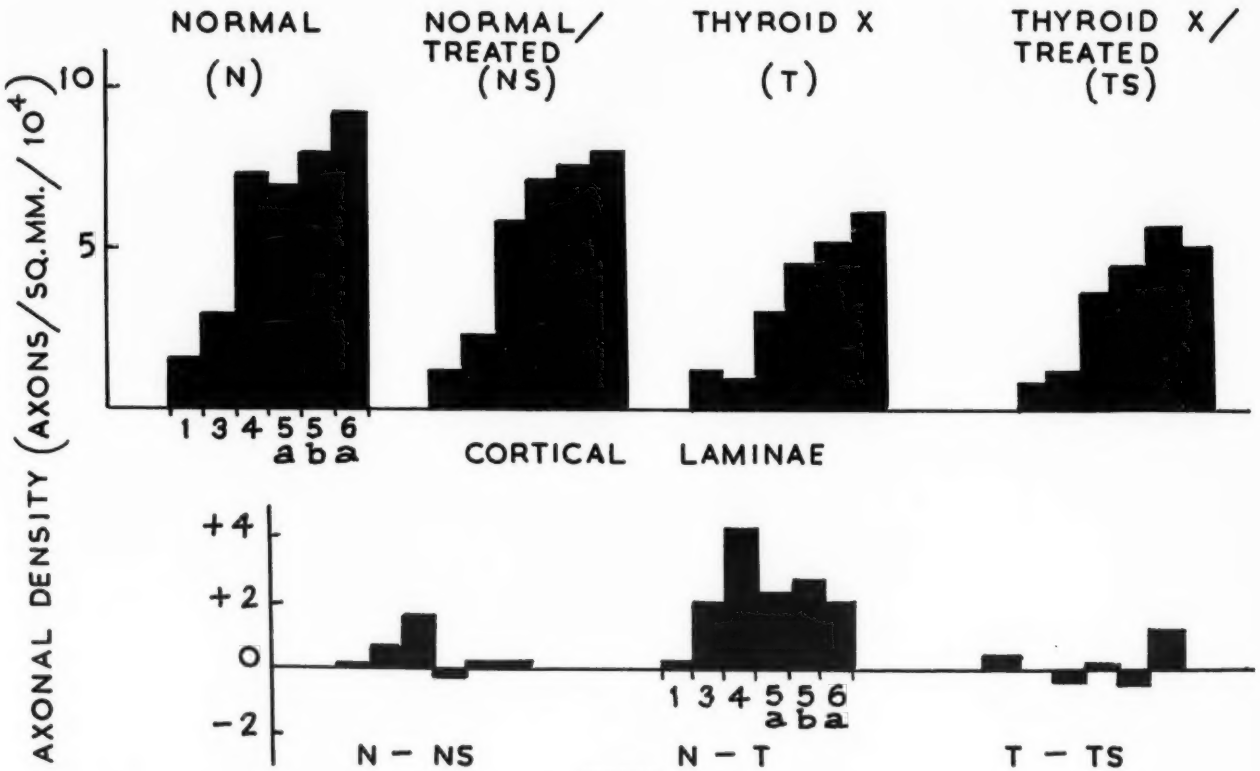

FIGURE 2

Effect of giving purified somatotrophin to normal and neonatally thyroidectomised rats on the development of the axon network in sensorimotor cortex.

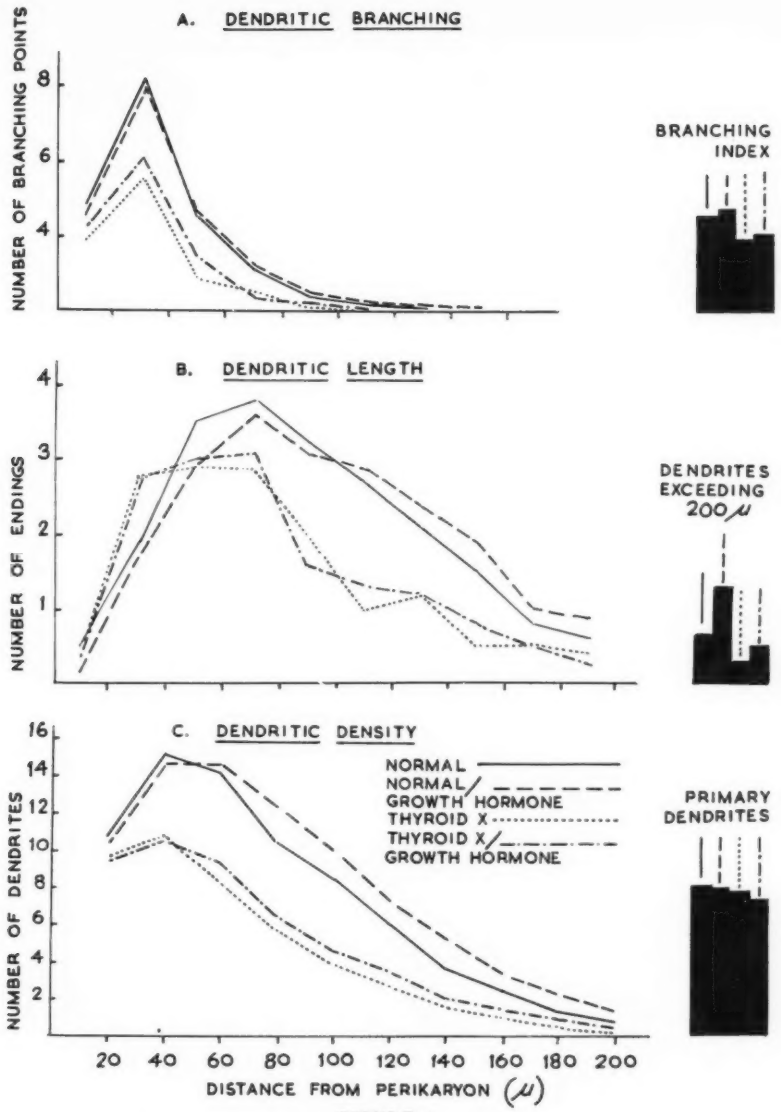

FIGURE 3

Effect of radiothyroidectomy and of giving purified somatotrophin on the growth and distribution of basal dendrites in layer 5b of the sensori-motor cortex of the rat. Primary dendrites are those emerging from the perikaryon: the branching index is the ratio between the number of primary dendrites and the total formed by branching.

the dendritic field (Fig. 3C). Combination of the data for axons and dendrites (Uttley, 1955) revealed a reduction in the probability of interaction between neurones from a factor of 296 in the normal animal to one of 123 in the thyroidectomised. This decrease, amounting to about 80%, was associated with a delay of 8.3 days in the time at which the placing reaction could first be elicited (Table 1).

TABLE I
EFFECT OF SOMATOTROPHIN ON AGE OF EMERGENCE OF PLACING REACTION AND PROBABILITY OF AXO-DENDRITIC INTERACTION

Class of rat	Age of emergence of placing reaction	Number of axo-dendritic contacts per neurone
Normal	18.7	296
Normal given growth hormone	17.0 days	333
Difference	1.7 days ($P = 0.01$-0.001)	37 (12.5%)
Thyroidectomised	27.0 days	123
Thyroidectomised given growth hormone	25.0 days	144
Difference	2.0 days ($P = 0.01$-0.001)	21 (17.1%)
Difference between normal and thyroidectomised	8.3 days ($P < 0.001$)	173 (58.5%)

2. Effect of giving somatotrophin.

The administration of somatotrophin was responsible for an enhancement of growth in both normal and thyroidectomised rats which was, however, of but minor proportions. Fig. 1, in which the effects of the experimental treatments on the dimensions of body and brain are compared, shows that the weights of body and brain and the length and width of the brain were slightly larger in normal rats given somatotrophin than in untreated animals. Of these, only the increase in brain weight, however, reached an acceptable level of statistical significance ($P = 0.05 — 0.02$), and the relationship between the weight of body and brain, together with that between the length and width of the brain were virtually unchanged. The effects of giving somatotrophin to neonatally thyroidectomised rats was similar

in trend but somewhat more marked (Fig. 1). Gains in the weights of both body and brain were statistically significant ($P = 0.05 — 0.02$ and < 0.001 respectively) and a significant fall of 7.5% in the brain/body weight ratio ($P < 0.01$) suggests that the amelioration in rate of growth favoured that of the brain more than that of the body. Both the width and length of the brain were significantly increased ($P < 0.01$) but in approximately the same proportion. Thus although there was a tendency towards a restoration of a normal length/width relationship this could not be regarded as significant statistically.

Figs. 2 and 3 illustrate the effects of giving somatotrophin on the growth of cortical neuropil. It is clear from Fig. 2 that this treatment was without consistent or significant effect upon the density of the axonal component either in the normal or thyroidectomised animal. On the other hand the data contained in Fig. 3 provide evidence for a hyperplasia of dendritic processes in which an increase in the length of dendrites plays a greater part than one of branching. Thus there was no significant change either in the mean number of dendrites arising from the perikaryon, or in the branching index i.e., the ratio between the number of primary dendrites and that ultimately formed by peripheral branching and there was little alteration in the pattern of distribution of branching points (Fig. 3A). An increase of 7.4% in mean dendritic length was however, observed in both normal and thyroidectomised rats given somatotrophin while the number of dendrites exceeding 200 μ in length was likewise increased in each case (Fig. 3B). This effect resulted in a shift to the right in the curves representing the densities of dendrites at successive distances from the centre of the perikaryon. The apparent lack of effect of the experimental treatment upon axonal densities must be interpreted in the light of this hypertrophy of the dendritic field; for an increase in the space occupied by dendrites would seem to imply that axons must have been absolutely increased in number though relatively unchanged in density. Combination of the data relating to both axons and dendrites revealed an increase in the probability of axo-dendritic interaction amounting to 11.1% in the normal and 15.7% in the thyroidectomised rats. This increase was associated with a significant advance in the age at which the placing reaction could first be elicited in both groups of rats (Table 1).

3. Effect of giving steroid anabolists.

The effects of giving MAD and 19NT were essentially similar. Both were responsible for a small reduction in the weight of the body and brain, but no material change in the linear dimensions of the brain or in their relative proportions could be detected. Axonal density was little altered in either normal or thyroidectomised rats given these steroids, and in view of these findings and those relating to the size of the brain the laborious task of estimating dendritic distribution was not undertaken in these animals.

Discussion

The results of these experiments, while by no means spectacular and partly negative in character, have been sufficient to provide at least partial answers to the two questions posed at the outset. In the first place it has been clearly shown that the effects of radiothyroidectomy are so strikingly similar to those induced by the administration of goitrogen (Eayrs and Taylor, 1951; Eayrs 1955) and, as far as can be judged by the more limited data available (Salmon, 1936, Scow and Simpson, 1945) or surgical thyroparathyroidectomy, as to minimise the possibility that they could be attributable to secondary influences such as, for instance, toxicity resulting from the chronic application of foreign substances or the concomitant involvement of the parathyroid. The view that thyroid secretion may be regarded as a potent factor determining the development and patterning of the cortical neuropil is thus strengthened.

The second question on which these results throw some light concerns the relative parts played by somatotrophin and thyroid hormone in regulating the incremental and maturational growth of the brain. Maturation may be taken as signifying those changes in bodily structure which, apart from mere increase in size, characterize the transition from infant to adult. Such criteria as have been used to distinguish maturational phenomena in so far as they relate to the growth of the body as have included the pattern of change occurring in the relative sizes, finer structure and composition of different organs, the nature of the pelage and dentition and in particular the pattern of skeletal growth (see Ray et al., 1950). From these, apart from gross measurement of size, changes in the growth of the central nervous system have largely been excluded and indeed, the relatively little

direct effect exerted by either thyroidectomy (Scow and Simpson, 1945) or hypophysectomy (Walker et al., 1952) upon the growth of the brain and the eyeball suggested that the development of these structures might to some extent be regulated by factors other than those which control that of the remainder of the body. Recent findings, however, have drawn attention to three abnormalities of cerebral development which, since they arise as a result of neo-natal hypothyroidism, may perhaps be regarded as reflecting the influence of maturational processes. These are (i) a failure on the part of the brain to maintain the normal properties of growth in width and length (Eayrs and Taylor, 1951), (ii) a selective retardation in the development of the axonal plexuses of layer 4 of the cerebral cortex and (iii) a departure of the mean dendritic from the pattern of exponential decay characteristic of the normal individual both of which may be attributable to a reduced capacity for branching on the part of cell process (Eayrs, 1955). The administration of growth hormone, although stimulating to a small extent the growth of the brain, failed to rectify any of these abnormalities and, in fact, the effect of this procedure in the thyroidectomised animal were little different from those in the normal. No differential increase in the amount of branching was seen in the brains of somatotrophin-treated thyroidectomised rats and the observation that intracortical connectivity was enhanced, and was associated with an advance in the time of emergence of the placing reflex, appeared largely to be due to an increase in the mean length of dendritic processes rather than to any marked change in capacity for branching. Similar findings have been reported in the young of females given purified somatotrophin during the later stages of gestation (Clendinnen and Eayrs, 1961). It is therefore reasonable to infer from the additional evidence now presented that the growth of the brain is influenced by somatotrophin rather more than might have been anticipated from earlier reports in the literature, but that this influence is, as is the case with organs of the body (Ray et al., 1950), related to the incremental rather than to the maturational aspects of development.

A striking, and somewhat unexpected, feature of the experimental findings has been the inability of any of the protein anabolic substances used to induce responses during the very early stages of postnatal development of an order comparable with those seen in

later life (e. g., Scow et al., 1949). Such observations are not without precedent, however, for Zamenhof (1942) reported a retardation in the growth of newborn young given a less pure form of somatotrophin than that used in the present experiments and Korner and Young (1955), have reported a similar effect in young male rats given MAD. The effects of these substances in the immature would therefore seem to differ significantly from those in the older animals which have been used to determine the growth-promoting properties of the substances in question (e. g., Scow et al., 1949; Ray et al., 1950; Korner and Young, 1955; Selgado and Selye, 1954). The reasons for this discrepancy are far from clear. One possibility is that of toxicity, or in the case of somatotrophin, of tissue reaction following the administration of a foreign protein. Dosage of each of the substances used however, though high, was based on that found by others to have marked anabolic effects in post-weanling and pubertal animals and in such subjects little or no toxic effect has been reported while the antibody response to a foreign protein might be expected to be less when administration is begun in the newborn than in the mature individual (e.g., Hanan and Oyama, 1954; Dixon and Maurer, 1955; Billingham and Brent, 1956). Moreover, a repetition of the present experiment using a considerably smaller dosage of somatotrophin gave strictly comparable results. An alternative explanation which might be advanced in the case of the steroid anabolists presupposes an inhibition by these substances of the gonadotrophic activity of the pituitary to such an extent that any direct growth promoting effect of exogenous steroid is outweighed by the depletion of endogenous androgen. There is little evidence with which to test this hypothesis, but it may be pointed out that MAD has few androgenic side-effects (Gordan, Eisenberg, Moon and Sakomoto, 1951) and hence might be expected to exert little inhibitory influence on the pituitary while 19NT, which was found by McSwiney and Prunty (1957) to be androgenic, proved to have marked oestrogenic properties in the newborn rat judged by the histological appearance of the gonads and reproductive tract of both male and female at post-mortem. No significant difference in the reaction of male and female to the experimental treatment was observed, a fact which argues against the view that the suppression of endogenous androgenic activity was involved. Perhaps the most likely explanation of the apparent refractoriness of the tissues to stimulation by somatotrophin is that the rate of growth this period is the

maximum compatible with such factors as the capacity for ingestion and assimilation of food and its conversion to tissue constituents and that until there is a reserve capacity for providing raw material no stimulus to the synthesis of protein can become effective. The relationship of the factors of age and state of development to the effectiveness of growth promoting substances clearly provides a field in which further study is needed.

Summary

The effects of neo-natal radiothyroidectomy on growth and cerebral development, and the influence of substances credited with the capacity to stimulate protein anabolism, have been measured in the rat.

The developmental changes seen in radiothyroidectomised animals were almost identical with those produced earlier by living goitrogen thus lending force to the argument that these are directly due to the absence of thyroid hormone.

Purified somatotrophin, while partially ameliorating the effects of thyroid deficiency in post-weanling rats, has a very weak influence during infancy and one which is no greater in thyroidectomised rats than in normal. The small changes in cerebral structure which resulted from its administration were better related to the incremental than to the maturational aspects of development.

The steroids methylandrostenediol and 19-nor-testosterone were without anabolic effects in the dosage given and even gave rise to catabolic tendencies.

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ERRATUM

To correct an error in Volume XXV, No. 1, Page 18: The last sentence should read "Figure 13 shows amputated portions of phalanges shown in Figure 12."